AGE-ASSOCIATED ALTERATIONS TO SPERM DNA METHYLATION AND POSSIBLE CONSEQUENCES ON THE OFFSPRING

by

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ABSTRACT

The effects of advanced paternal age have received little attention in the past. However, recent data from multiple groups suggesting an association between advanced paternal age and various disorders in the offspring have sparked a great deal of interest. Specifically, offspring of older fathers have an increased risk of autism, bipolar disorder, and schizophrenia among other issues. Further fueling this interest is the striking trend of delayed parenthood in developed countries, likely a result of socioeconomic pressures and increasing divorce rates with subsequent remarriage. Increased interest in the "paternal age effect" has driven some private tissue banks to promote cryopreservation of sperm at a young age to avoid the detrimental impacts of aging. Taken together, there is a great need for further investigation into this phenomenon.

Although there is solid epidemiological evidence to substantiate the impacts of advanced paternal age on the offspring, the etiological mechanisms that drive this process remain poorly defined. Among the most plausible contributing factors to the increased incidence of various diseases in the offspring of older fathers are age-associated alterations to the sperm epigenome. Specifically, sperm DNA methylation perturbations may have the capacity to affect offspring phenotype through transcription inhibition/activation but have yet to be explored in the context of advancing age.

To investigate age-associated sperm DNA methylation alterations, we have analyzed sperm from 17 donors who collected samples between 9 and 19 years apart.

DNA methylation was analyzed in all samples at both the global and cytosine phosphate guanine (CpG) level. The results indicate that global sperm DNA methylation increases with age while regional alterations are strongly biased toward hypomethylation, a finding in stark contrast to somatic cell age-associated methylation alterations. Intriguingly, many of the genes affected by regional methylation alteration have been previously suggested to be associated with many types of neuropsychiatric disorders, including schizophrenia, bipolar disorder, developmental delay, personality disorders, and autism.

These data provide the first direct evidence that age-associated epigenetic alterations to the sperm represent a plausible mechanism for the increased incidence of various abnormalities in offspring of older fathers.

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The following chapter is a reprint of an article coauthored by myself and Carrell, DT. It was originally published in *Reproduction*, Volume 143, pages 727-734, 2012 (copyright by Society for Reproduction and Fertility). Reprinted with permission.

REPRODUCTION
REVIEW

The sperm epigenome and potential implications for the developing embryo

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Abstract

Recent work in the field of male fertility has yielded significant increases in our understanding of the sperm epigenome and its potential role in embryonic development. These new findings have enabled a broad classification of a normal epigenetic state in the male gamete and have provided insight into the possible etiologies of some idiopathic male infertility cases. Histone retention and modification, protamine incorporation into the chromatin, DNA methylation, and spermatozoal RNA transcripts appear to play important roles in the epigenetic state of mature sperm. These epigenetic factors may reveal a historical record of spermatogenesis, portend future functions in embryogenesis, and help to elucidate mechanism of pluripotency. In contrast to the once held dogma regarding the importance of the paternal epigenome, the unique epigenetic landscape in sperm appears to serve more than the gamete itself and is likely influential in the developing embryo. In fact, growing evidence suggests that mature sperm provide appropriate epigenetic marks that drive specific genes toward activation and contribute to the pluripotent state of the embryonic cells. Although not definitive, the current literature provides evidence for the role of the sperm epigenome in the embryo. Future work must be focused on the characterization of epigenetic abnormalities commonly found in individuals with compromised fertility to further establish this role. Additionally, studies should target the effects of environment and aging on the sperm epigenetic program and subsequent fertility loss to determine the etiology of aberrant epigenetic profiles.

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Introduction

The sperm epigenetic program is unique and tailored to meet the needs of this highly specialized cell. Chromatin changes in sperm contribute to virtually every function that the male gamete must perform throughout spermatogenesis and in the mature cell. One requirement of sperm is that of transportation through both the female and male reproductive tracts, which demands a highly motile cell type. The unique nuclear protein landscape in sperm creates a chromatin structure that is between six and 20 times more dense than nucleosome-bound DNA, ultimately resulting in tightly condensed nucleus (Ward & Coffey 1991, Balhorn 2007). The compacted sperm head is proposed to provide enhanced motility and protection from DNA damage in a cell type that lacks robust repair mechanisms (Oliva & Dixon 1991). This is particularly important as sperm encounter many harsh environments during transport through the female reproductive tract.

The unique chromatin structure in sperm is essential for the safe delivery of paternal DNA to the oocyte, but the requisite replacement of canonical histones with sperm-specific protamine proteins has called into question the utility of the paternal epigenome in embryonic development. The stepwise transition of sperm nuclear proteins first involves the replacement of canonical histones with transition proteins. Next, two isoforms of protamine proteins, protamine 1 (P1) and P2, take the place of transition proteins in the sperm chromatin. The ratio of incorporated P1 and P2 is tightly regulated at ~1:1 ratio in the mature sperm (Balhorn *et al*. 1988, Hecht 1990, Oliva & Dixon 1990, Dadoune 1995). Aberrations in this ratio have been correlated with general infertility and poor fertilization ability (Aoki *et al*. 2005, 2006, Zhang *et al*. 2006). The protamination of sperm chromatin provides the compaction necessary for safe delivery to the oocyte but removes histones, which are capable of eliciting gene activation or silencing via tail modifications (methylation, acetylation, etc.). In effect, protamination removes a potentially informative epigenetic layer from the paternal chromatin, leading to the previously held belief that sperm are incompetent to

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drive epigenetic changes in the embryo and suggesting that their utility is found only in the delivery of an undamaged DNA blueprint. However, recent data challenges this dogma.

It is known that the replacement of histone with protamine is incomplete, with about 5–15% of the chromatin remaining nucleosome-bound (Tanphaichitr *et al*. 1978, Wykes & Krawetz 2003). Interestingly, this incomplete replacement was found to be programmatic and not simply a result of inefficient machinery, suggesting that retained histones may contain modifications that play a role in epigenetic regulation (Arpanahi *et al*. 2009, Hammoud *et al*. 2009). In known fertile patients, histone retention is found at the promoters of genes important in the embryo including developmental gene promoters, microRNA clusters, and imprinted loci, suggesting that the nucleosome retention is programmatic in nature (Hammoud *et al*. 2009). Taken together, these data suggest that the limited view of the sperm epigenome in developmental regulation is incomplete.

The growing evidence in support of the hypothesis that the paternal epigenome plays an important role in the developing embryo is not limited to nucleosome retention data. Recent studies analyzing sperm DNA methylation, histone modifications, and spermatozoal RNA transcripts further establish the role of the sperm epigenetic program in the developing embryo. This review will describe the current literature regarding the paternal epigenome and its influence on embryogenesis and will additionally address critical future research directions.

DNA methylation

DNA methylation is a common regulatory mark found on the 5 carbon of cytosine residues (5-mC) at cytosine–phosphate–guanine dinucleotides (CpGs; Fig. 1), which exert strong epigenetic regulation in many cell types (Portela & Esteller 2010). DNA methylation is essential in genomic imprinting, gene expression regulation, X chromosomal inactivation, and embryonic development (Jaenisch & Jahner 1984, Surani 1998, Ng & Bird 1999). This epigenetic mark can activate or repress gene transcription at specific sites based on the methylation levels at promoter regions. Hypermethylation at promoters blocks access of transcriptional machinery and thus inhibits gene expression. Conversely, hypomethylation facilitates gene activation as a result of increased accessibility of DNA by polymerase (Fig. 2).

The regulation of DNA methylation is essential to normal cell function in somatic cells, gametes, and the embryo (Jaenisch & Jahner 1984, Surani 1998, Ng & Bird 1999). The DNA methyltransferase (DNMT) family of proteins helps to facilitate *de novo* methylation and methylation maintenance (Eden & Cedar 1994). The enzymes directly responsible for *de novo* methylation include DNMT3A (DNMT3a), DNMT3B (DNMT3b), and DNMT3L (DNMT3l). Both DNMT3A and DNMT3B contain catalytic domains, which allow them to directly lay down new methylation marks. DNMT3L is essential in directing the proper placement of marks by working in concert with DNMT3A and DNMT3B (Okano *et al*. 1999). Once methylation marks have been established, DNMT1 maintains those marks through cell division (Bestor 1992, Lei *et al*. 1996).

The importance of DNA methylation has been demonstrated globally, regionally, and at the single locus level in both humans and animal models. Of great value in the effort to increase our understanding of the role of DNA methylation is recent data describing the human sperm methylome, which provides a general classification of a 'normal' methylation status at 96% of genomic CpGs (Molaro *et al*. 2011). Recent data demonstrate that aberrant methylation of promoters for specific genes (e.g. *DAZL* and *MTHFR*) and general gene classes, such as imprinted loci, are strongly associated with various forms of infertility and sperm defects in men

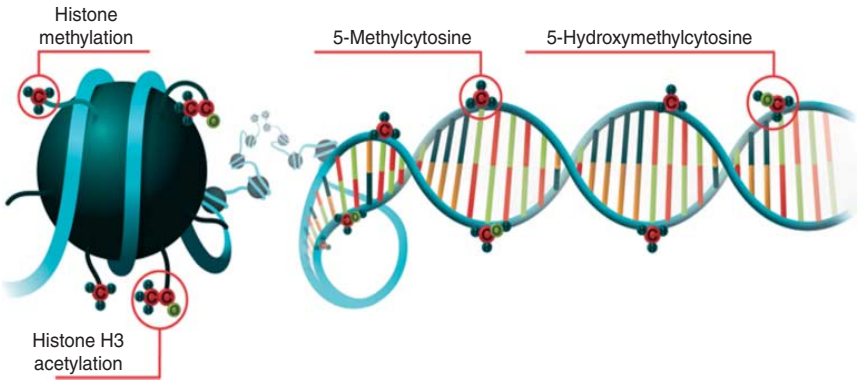

Figure 1 Epigenetic modifications (both histone modifications and DNA methylation) commonly found in sperm. Depicted are histone tail modifications (methylation and acetylation) as well as 5-methylcytosine (5-mC) and the demethylation intermediate, 5-hydroxymethylcytosine (5-hmC). Each modification is believed to play a regulatory role in gene expression.

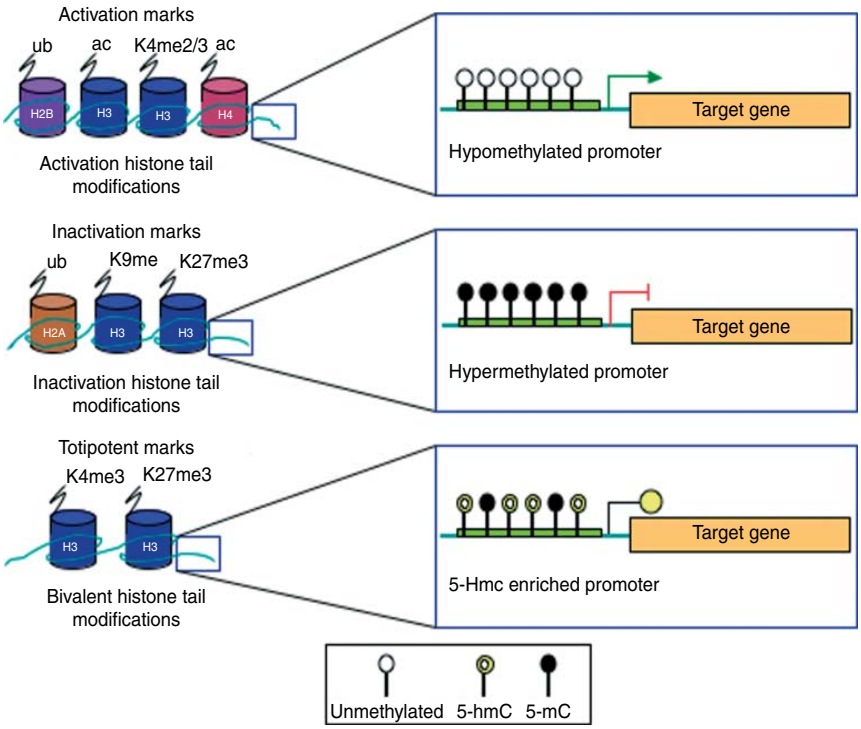

Figure 2 This figure depicts epigenetic marks that contribute to gene activation or inactivation, as well as bivalent modifications. Epigenetic regulators that promote gene transcription include DNA hypomethylation, histone H2B ubiquitination, H3 acetylation, H3K4 methylation, and H4 acetylation. Marks that tend to inhibit transcription include DNA hypermethylation, H2A ubiquitination, H3K9 methylation, and H3K27 methylation. Bivalent histone modifications (H3K27 methylation and H3K4 methylation) and enrichment of 5-hmC at gene promoters appear to be a hallmark of pluripotency.

(Navarro-Costa *et al.* 2011, Pacheco *et al.* 2011, Wu *et al.* 2011). Additionally, El Hajj *et al.* (2011) suggest that improper methylation of repetitive elements may be linked to recurrent pregnancy loss. Methylation abnormalities at the promoter of CREM were seen in a subset of patients with coinciding protamine ratio abnormalities as well as in patients presenting with various forms of male factor infertility (Nanassy & Carrell 2011*a*, 2011*b*). Additionally, Benchaib *et al.* (2005) reported that among IVF patients, global sperm DNA hypomethylation is correlated with poor pregnancy outcomes. Interestingly, the data revealing the importance of DNA methylation are not only limited to studies on patients with reduced fertility but has also been suggested on known fertile donors. These data reveal an epigenetic landscape that appears programmed for use in the embryo with hypomethylated DNA at developmental promoters (Hammoud *et al.* 2009). Taken together, these data suggest that DNA methylation may play an essential role in both the sperm and the embryo.

Multiple targeted studies have been performed in animal models to establish a clear role of DNA methylation in the sperm and embryo. Knockouts and mutations of *Dnmt1* in mice caused severe global hypomethylation resulting in biallelic expression at imprinted differentially methylated regions (DMRs), transcription of retrotransposons, loss of chromosomal inactivation, retarded gestational growth, and, in turn, embryo lethality (Li *et al.* 1992, Panning & Jaenisch 1996, Walsh *et al.* 1998). Conditional knockouts of DNMT3A and DNMT3L were hypomethylated at imprinted DMRs and resulted in severe decreases in spermatogenesis with DNMT3L loss also resulting in global decreases in methylation (Kaneda *et al.* 2004, La Salle *et al.* 2007). The loss of DNMT3B did not result in severe phenotypes, suggesting that there is redundancy in its function. However, the loss of DNMT3B did prove to affect *Rasgrf1* promoter methylation, which was not seen in the DNMT3A conditional knockout (Kaneda *et al.* 2004, Kato *et al.* 2007). These data suggest that there are regions where DNMT3A and DNMT3B act independently, as well as regions of redundancy.

In addition to studies using targeted genetic approaches in the study of DNA methylation are those using 5-azacytidine and 5-aza-2-deoxycytidine, both potent DNA methylation inhibitors (Egger *et al.* 2004). Short-term 5-aza-2-deoxycytidine exposure in neonatal male mice reduced overall fertility status by halting spermatogenic differentiation (Raman & Narayan 1995). Adult male mice treated with 5-azacytidine before

mating had decreases in fertility and increased incidence of embryo mortality (Seifertova *et al*. 1976). Additionally, 5-aza-2-deoxycytidine treatment in both mouse and rat resulted in decreased fertilization rates and/or increased preimplantation loss (Doerksen & Trasler 1996, Kelly *et al*. 2003, Oakes *et al*. 2007). It should be noted that while global demethylation is observed in the sperm with this treatment, the effects on embryogenesis may ultimately be a result of the cytotoxic effects of the drug and not solely the result of methylation defects (Oakes *et al*. 2007).

Along with the current interest in CpG methylation, recent data has suggested that intermediates formed during DNA demethylation may be important epigenetic regulators. Most prominent among these intermediates is 5-hydroxymethylcytosine (5-hmC; Fig. 1). This DNA modification is formed via the TET family of proteins (Tahiliani *et al*. 2009) just before complete removal of methylation marks. Because it is an intermediate, 5-hmC can achieve full demethylation more quickly than 5-methylcytosine, and as a result, gene promoters with enriched 5-hmC are considered easier to activate and thus may play a role in epigenetic regulation (Fig. 2; Pastor *et al*. 2011). Interestingly, recent work from Pastor *et al*. (2011) has revealed an enrichment pattern of 5-hmC at poised genes in the stem cell. This unique localization suggests that 5-hmC has utility in the embryonic stem cell and possibly the epigenome of multiple other cells types. As nucleosome retention was found at similar poised loci in sperm (Hammoud *et al*. 2009), identification of regional 5-hmC enrichment must be analyzed in the male gamete. These data suggest that 5-hmC may have real impacts on the epigenome and must be a focus of future studies to fully elucidate its regulatory role.

The current literature provides strong evidence for the importance of appropriate DNA methylation. This essential epigenetic mark has tremendous utility in the maintenance of gene activation and repression. Inappropriate methylation clearly results in abnormal phenotypes and as such, efforts should be made to determine the etiology of these aberrant profiles so that proper preventative steps and treatments can be established.

Retained histones, their modifications, and spermatozoal RNA transcripts

One of the most unique epigenetic features in the male gamete is the replacement of DNA-bound histones with protamines. As mentioned earlier, this protamination creates a highly condensed nuclear structure that helps to enable proper motility and protects DNA from damage. Although incorporation of this unique, sperm-specific protein results in a quiescent chromatin structure, some regions retain histones and their associated modifications. Recent studies have found this nucleosome retention to be programmatic and not a result of random distribution (Arpanahi *et al*. 2009, Hammoud *et al*. 2009). The mechanism that directs this selective nucleosome retention remains largely uncharacterized, but there is evidence to suggest a role for RNA transcripts in the process (Rassoulzadegan *et al*. 2006, Dadoune 2009). It appears that spermatozoal RNA transcripts are capable of inhibiting the protamination process and maintaining a histone-bound chromatin structure (Miller *et al*. 2005). RNA transcripts colocalize with nucleosome-bound chromatin near the nuclear envelope in the mature sperm, as is the case with the insulin-like growth factor 2 (IGF2) locus (Wykes & Krawetz 2003, Miller *et al*. 2005). There remains controversy in the role of RNA in this process, but the colocalization with regions of retained nucleosomes does provide a possible mechanism to explain the regulation of histone retention. In theory, this selective retention in sperm could allow for targeted gene activation or silencing in the embryo.

Multiple histone variants found in sperm play an essential role throughout spermatogenesis as well as in the mature spermatozoa. Among these, important nuclear proteins are histone 2A and B (H2A and H2B), histone 3 (H3), histone 4 (H4), and the testes variant (tH2B) (Gatewood *et al*. 1990, Jenuwein & Allis 2001, Fenic *et al*. 2004, Baarends *et al*. 2005, Zhu *et al*. 2005, Okada *et al*. 2007). Histone proteins have the distinct capability of driving epigenetic changes based on tail modifications. As a result, those histones retained through the protamination process are likely competent to exert similar regulatory effects. Targeted gene activation or silencing in many different cell types can be driven by these tail modifications found at lysine (K) and serine (S) residues of histones. The main forms of modifications in sperm include methylation, acetylation, ubiquitination, and phosphorylation, which can act alone or in concert to ensure the proper state of activation or suppression at any given gene or gene promoter (Fig. 1). H3K4 methylation, H3 and H4 acetylation, and H2B ubiquitination drive the genes toward activation while H3K9 and H3K27 methylation, deacetylation at H3 and H4, and H2A ubiquitination enrichment support gene silencing (Fig. 2; Jenuwein & Allis 2001, Lachner & Jenuwein 2002, Baarends *et al*. 2005, Zhu *et al*. 2005). These modifications are established and regulated by a variety of enzymes. The histone methyltransferase and demethylase family of proteins catalyze methylation and demethylation (Lachner & Jenuwein 2002). Acetylation establishment and removal are regulated by histone acetyltransferase and deacetylase respectively (Jenuwein & Allis 2001).

As a result of protamination, few histones remain to function as epigenetic regulators. However, as previously mentioned, the few loci that have been shown to retain histones in the mature sperm are known

to be important in developmental processes (Hammoud *et al*. 2009). This suggests that histones in these select regions are able to provide some degree of retained regulatory competence via histone tail modifications. Indeed, recent studies have implicated aberrant histone methylation and/or acetylation in the mature sperm in various forms of infertility. Loss-of-function mutation of JmjC-domain-containing-histone demethylase 2A (JHDM2A), an enzyme with known H3K9 demethylase activity, revealed decreased transcription of transition protein 1 and P1 during spermatogenesis (Okada *et al*. 2007). Additional studies have demonstrated that varied degrees of infertility, including sterility, are correlated with perturbations in histone methylation (Lee *et al*. 2005, Glaser *et al*. 2009). Deacetylase inhibitors, such as trichostatin-A, have been used in the study of histone modification and epigenetic regulation in mice. Fenic *et al*. (2004, 2008) demonstrated that s.c. injection of trichostatin-A in male mice resulted in a dose-dependent decrease in spermatogenesis and testis weight as well as decrease in histone deacetylase activity and subsequent alterations in histone acetylation. On a more broad level, recent work from Hammoud *et al*. (2011) has shown that histone retention is not programmatic but random in some patients with two different classes of infertility. A decreased enrichment of H3K4me or H3K27me at select regions important to development was also identified. These studies provide additional evidence that suggests a regulatory role of histone modifications in sperm.

In normal human sperm, histone modifications and their enrichment patterns suggest a highly regulated epigenetic landscape. H3K4 dimethylation (H3K4me2) and H3K4 trimethylation (H3K4me3) were found enriched at developmental promoters important in the embryo. Additionally, H3K27 trimethylation (H3K27me3) is enriched at gene promoters that are silenced in the early embryo (Hammoud *et al*. 2009). Further data describe a pluripotent-like state that some genomic regions important to the developmental program show in both human and mouse (Hammoud *et al*. 2009, Brykczynska *et al*. 2010). In these regions, bivalent histone modifications, H3K4me3 and H3K27me3, reflect marks found in stem cells (Fig. 2). These findings provide additional evidence that the paternal epigenome may be needed for proper embryonic development and may additionally contribute to the pluripotent state of embryonic stem cells.

Taken together, these data suggest a role of the paternal epigenome in early embryonic development. Nucleosome retention provides additional epigenetic competence to the paternal chromatin that was once considered to be void of such marks. It appears likely that histone retention and histone modifications are key to normal sperm function and ultimately normal embryogenesis. As our understanding of the role of histones and their modifications in sperm increase, we will be able to better classify, diagnose, and treat male factor infertility.

Conclusions and future directions

The role of the paternal epigenome in the embryo has long been considered to exert only limited influence on development. However, the recent literature described in this review provides evidence that the unique epigenetic landscape in sperm may play a larger role in development than previously believed. From these data, it is evident that proper establishment and maintenance of the paternal epigenetic program is associated with appropriate gamete and embryonic development, disruption of which is associated with varied degrees of infertility. While recent studies have been key in opening this relatively new area of study, many questions remain unanswered.

Further research is required to fully elucidate the paternal regulatory control in the embryo. One key will be to further investigate the epigenetic profiles of various classifications of infertility along with embryo quality data. Special caution must be taken to ensure that proper patient classification is used to ensure that results are clearly attributable to unique and definable etiologies as opposed to infertility in general. This will allow a determination of direct correlations, and possibly causation, to specific infertility classes.

In addition to observing aberrant epigenetic profiles common among specific populations of infertile men, the etiologies of these various abnormalities must be investigated. There are many likely candidates that may cause epigenetic alterations in sperm and resultant abnormal embryogenesis, the most prominent of which are environmental toxins and aging. Studies have evaluated various environmental agents and their effects on male fertility in general, but few have analyzed specific epigenetic alterations that may be occurring as a result of exposure. However, there is precedence for environmental impacts on the paternal epigenetic landscape. Recent studies provide intriguing evidence for the effects of heavy metals on sperm nuclear proteins, specifically P2. Quintanilla-Vega *et al*. (2000*a*, 2000*b*) reported that lead binds to P2 and inhibits its DNA binding, ultimately affecting chromatin compaction. Yauk *et al*. (2008) found that mice exposed to ambient air pollution had global DNA hypermethylation compared with animals housed with high-efficiency particulate arresting (HEPA)-filtered air. Additionally, exposure to endocrine disrupters has also been shown to disrupt heritable germ cell epigenetics (Anway & Skinner 2008). These data clearly suggest that environmental agents can affect sperm epigenetics. Environmental toxins must be targeted in future studies analyzing the etiology of epigenetic alterations in sperm to aid improvement in diagnostic and treatment approaches in men who present with infertility.

In addition to the effects of the environment on the sperm epigenome, there is also increasing concern regarding the effects of advanced paternal age on epigenetic alterations seen in male gametes. It is well established that advanced maternal age is associated with poor pregnancy outcomes, mainly as a result of increased incidence of chromosomal nondisjunction (Eichenlaub-Ritter 1998). Conversely, paternal aging has long been considered of little consequence in the development of functional sperm capable of generating normal offspring. However, the available data suggest that there are likely aberrations occurring in the sperm of aging males that may affect offspring. Recent work from Flatscher-Bader *et al*. (2011) has shown that copy number variations are more common in the offspring of older male mice than from younger fathers. Although the idea of epigenetic alterations occurring as a result of aging in males is still controversial, recent studies have yielded interesting data on this front as well. Changes in gene expression and chromatin compaction in sperm and testes tissue of older males have been described in recent studies while contradictory data have been presented in other studies (Wyrobek *et al*. 2006, Zubkova & Robaire 2006, Kokkinaki *et al*. 2010). Despite some controversy when taken together, these data still appear to suggest that advanced paternal aging may be accompanied by some degree of epigenetic alterations in the sperm. These changes are, however, poorly characterized.

It is evident that the paternal epigenome plays a role in sperm quality and likely in embryonic development; however, many unanswered questions remain. It will be critical for future studies to focus on common epigenetic abnormalities found in specific classes of infertility. Additionally, research should focus on the etiologies of such abnormalities. The effect of environmental exposures and aging are two major topics that have yet to be fully addressed in relation to the genesis of an aberrant sperm epigenetic program. As we learn more about the true effects of epigenetic alterations in sperm, how they arise and how they affect fecundity, we will be more capable of addressing the growing issue of male factor infertility in prevention, diagnosis, and treatment.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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CHAPTER 2

THE AGING MALE AND IMPACT ON OFFSPRING

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3 The Aging Male and Impact on Offspring

Timothy G. Jenkins, Kenneth I. Aston, and Douglas T. Carrell

3.1 Introduction

Advanced paternal age has become a heavily investigated topic recently as a result of multiple studies demonstrating ties between advanced paternal age and various offspring abnormalities. Further contributing to the increasing interest in the role of advanced paternal age in reproduction is the trend of delayed parentage believed to be a result of socioeconomic pressures in developed countries [1]. Though this trend is justified by increasing life expectancies in both sexes, advanced paternal age significantly affects general semen parameters and sperm quality that ultimately alters fecundity and may additionally affect offspring health. While many couples consider the risks associated with advanced maternal age in family planning decisions, very little thought is given to the age of male partners. As a result, it is important that physicians consulting couples with an aged male partner have the available data to help patients make well-informed family planning decisions based on the risks associated with advanced paternal age. This chapter will outline what is currently known regarding the effects of paternal age on fecundity and will also discuss the associations between advanced paternal age and the offspring's disease risk. These effects, based on current data are summarized in Table 3.1.

3.2 Delayed Parenthood

In recent history, the age of parenthood for both males and females has steadily increased in many developed countries. This trend is believed to be associated with increased life expectancy, socioeconomic pressures, and divorce rates with subsequent remarriage at older ages [2]. During a 10-year span (1993–2003) in Great Britain, the percent of fathers who were in the age range of 35–54 increased from 25 % of total births to 40 %. Associated with this trend was a decrease in the number of births to fathers less than 35 years of age from 74 % of total births to only 60 % [3]. In Australia, over two decades (1988–2008), the

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Table 3.1 The effects of advanced paternal age on semen parameters and offspring disease risk

Parameter	Effect
Semen parameters	
Semen volume	↓
Sperm count	↓?
Sperm motility	↓
Sperm morphology	↓
Genetic/epigenetic	
DNA damage	↑
Aneuploidy rates	
Sex chromosomes	↑
Autosomes	~
Mutations	↑
Telomere length	↑
Chromatin packaging	Δ
Global methylation	Δ
Pregnancy rate	
Natural conception	↓
Insemenation	↓?
IVF	↓?
Offspring disease risk	
Autosomal dominant disorders	↑
Trinucleotide repeat disorders	↑
Cancer	
Hematologic	↑
Brain tumors	↑
Breast	↑
Prostate	↑
Neuropsychiatric disorders	↑

↓, decline; ↑, increase; Δ, change; ~, no change; ?, data are ambiguous

average age of fathers has increased by approximately 3 years [4]. Similarly, the average age of fathers in Germany increased by 2 years over a 10-year period [2]. Similar trends can be found in the United States and many other developed countries. As average paternal age continues to increase in many countries it is becoming increasingly important to characterize the potential consequences of advanced paternal age on fertility and offspring health.

3.3 Age-Related Changes in Sperm Quality

With advancing male age, a number of changes occur to sperm and semen that can impact fertility status or increase the risk of disease transmission to offspring. These changes include declines in some semen parameters, increased sperm DNA damage, genetic changes in sperm resulting from mitotic or meiotic errors or errors that arise during DNA replication, and epigenetic changes to sperm DNA. These changes are discussed below.

3.3.1 Changes in Semen Parameters

Unlike females, who are born with a finite number of gametes that are generally exhausted between the age of 45 and 55 years, coincident with menopause, men continue to produce sperm throughout their lives. While spermatogenesis continues well into old age, some semen parameters do decline as men age. Numerous studies have evaluated the effects of male age on semen parameters, but shortcomings of some of the individual studies include small sample size and failure to control for potentially confounding factors. For this reason there exists a significant degree of discordance between studies, making the reliable estimate of age effects difficult to quantify. However, a thorough review of the literature from 1980 to 1999 by Kidd et al. evaluated the effect of age on semen parameters and concluded that there is general agreement among studies that semen volume, sperm motility, and proportion of morphologically normal sperm all decline with advancing age [5]. These conclusions were corroborated by more recent literature reviews and carefully controlled primary research [6–8].

From the available literature, it can be inferred that semen volume significantly decreases with age, with a decline of 3–22 % from age 30 to age 50 [5, 8]. Similarly, a 3–37 % decrease in sperm motility is estimated to occur over the same period, as indicated in several studies [5, 8]. Finally, the best estimates for declines in normal sperm morphology indicate a decrease of 4–22 % between the ages of 30 and 50 [5, 8]. The data regarding changes in sperm concentration with age are less conclusive, and total sperm count has rarely been evaluated. Of more than 20 studies that evaluated the effect of male age on sperm concentration, there is essentially an even split between studies that report a decline, those that report no age effect, and those that report increased

sperm concentrations with advancing age [5, 8]. As semen volume significantly declines with age, if spermatogenic output remained constant, then sperm concentration would necessarily increase in older men. A recent study of 1,174 men age 45 and older reported a non-significant increase in sperm concentration with age, and a significant decline in total sperm count with advancing age in men between the ages of 45 and 80 [9].

While the consensus based on large datasets is that semen volume, sperm motility, and normal sperm morphology decrease with advancing age, the decreases are generally modest. Moreover, the number of confounding variables such as lifestyle factors, environmental influences, health status, abstinence periods, and others make it nearly impossible to identify the age-associated causes that are directly responsible for these declines.

3.3.2 Genetic Changes

The molecular hallmarks of aging throughout the body include increased oxidative damage, increased aneuploidy rates and chromosomal rearrangements, the accumulation of mutations within the genome, and telomere shortening [10, 11]. Sperm are particularly prone to many of these changes due to the high rate of cell division relative to most other cells types in the body. However, unlike telomere attrition that occurs in the majority of other cell types, the telomeres length in sperm actually increases with age. Genetic changes to sperm are discussed in the following section.

3.3.2.1 DNA Damage

Numerous studies have reported an age-related increase in sperm DNA damage [12–16]. The increase in DNA fragmentation index (DFI) is marked, with a nearly fourfold increase in men age 60–80 compared with men age 20–29 reported in one study [14]. In a large study of 1,125 men from infertile couples, DFI more than doubled in men over the age of 45 compared with men aged 30 and younger [16]. The mechanisms responsible for increased sperm DNA damage in older men are not completely characterized, but increased reactive oxygen species (ROS) [17], coupled with the insufficiency of DNA repair and apoptotic machinery, have been proposed [18].

3.3.2.2 Aneuploidy Rates

The increase in gamete aneuploidy rates in women with advancing age is well documented and dramatic. It is estimated that about 20 % of human oocytes are aneuploid, and the incidence has been reported to be as high as 60 %, with a sharp increase in the decade preceding menopause [19–21]. In contrast, sperm aneuploidy rates are much lower with an estimated average incidence of 1–2 % [20], and the effect of male age on sperm aneuploidy rate remains unclear. Some studies have failed to find an effect of male age on sperm aneuploidy frequency [14, 22], while others have reported a modest increase in aneuploidy rates related to age, particularly increased disomies of the sex chromosomes [23–25].

While there is no consensus on the effect of male age on sperm aneuploidy rates, the majority of evidence suggests a slight increase in sex chromosome disomy rates in older men and a general lack of an effect or a weak effect in the autosomes [8].

3.3.2.3 Increased Mutations

The introduction of de novo mutations into the genome is the basis for heritable genetic variation, and the number of mutations per genome is related to the number of replication cycles that a cell undergoes, as there is an error rate inherent in replication machinery. Based on family-based sequencing and single sperm sequencing as well as evolutionary measures, the de novo mutation rate of sperm is estimated to be between 1 and 4 changes per 100 million bases per generation [26, 27], while the mutation rate per cell division is almost three orders of magnitude lower than the per generation mutation rate [28]. The more cycles of DNA replication and cell division a cell undergoes, the greater the chance for mutations to occur in that cell. In women, from the primordial germ cell stage to ovulation, an oocyte will have undergone approximately 24 cell divisions [29]. In men that number is estimated to be approximately 30 cell divisions at puberty, with one spermatocyte cell division every 16 days, or 23 divisions per year after puberty (see Fig. 3.1) [29].

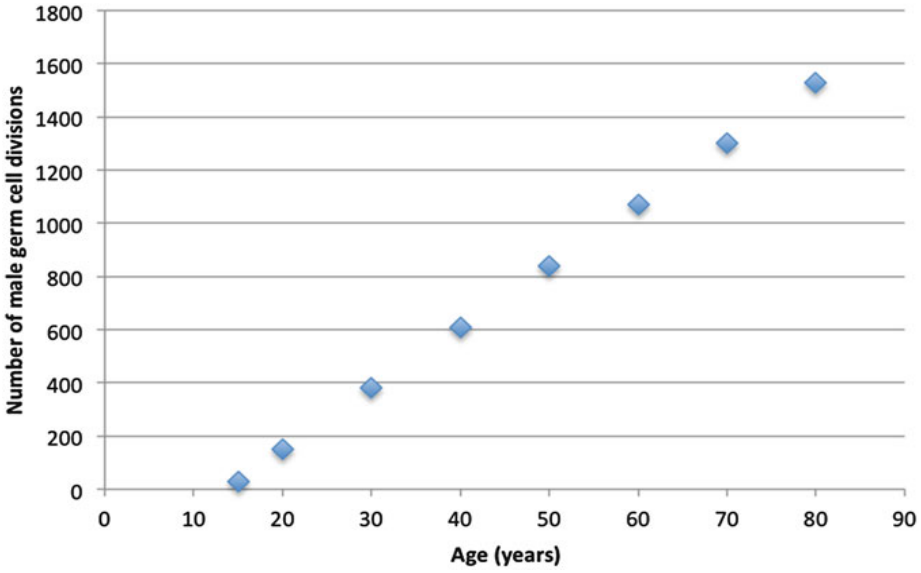

Fig. 3.1 Illustration of the estimated number of male germ cell divisions as a function of age

Clearly there is a greater opportunity for mutations to arise in sperm than in oocytes, and male age is predicted to be a strong contributing factor. Lionel Penrose was the first to propose a relationship between male age and mutations in offspring [30]. While the mutation load of individual sperm as a function of male age has not been directly measured, molecular genetics predicts that sperm from older men will, on average, harbor more mutations than sperm from younger men. This prediction is substantiated by a recent study of genomic sequence in parent–offspring trios that estimated an increase of approximately two mutations per year of paternal age [31]. In addition, the increased rates of specific autosomal dominant diseases and disease-specific mutation analysis also support an age effect on sperm mutation frequency [14], as will be discussed in detail below.

3.3.2.4 Changes in Telomeres

While the consequences of advanced paternal age on the genetics of sperm are generally negative, the age-related changes to sperm telomeres might confer some advantage to offspring. Telomeres are composed of long tracts of TTAGGG repeats located at the ends of each chromosome and serve as a buffer to the loss of important genetic material due to the inability of DNA replication machinery to replicate DNA at the very end of each chromosome. In addition, the telomere cap at the end of each chromosome distinguishes chromosome ends from double strand breaks and thus serves to protect against spurious chromosomal fusion [32]. While in most tissues, telomeres progressively shorten with age, ultimately resulting in cell cycle arrest or apoptosis, the telomeres in sperm are longer in older men [33], and children of older fathers have longer leukocyte telomeres than do children of younger fathers [34, 35]. Telomere inheritance may represent an example of a genetic advantage of delayed reproduction in men as longer leukocyte telomere length is associated with decreased risk of atherosclerosis and increased lifespan [36].

3.3.3 Epigenetic Changes

The effect of advanced paternal age on offspring has begun to receive much attention. Recent studies have linked paternal aging and the prevalence of well-known neuropsychiatric disorders in offspring [37–39]. Large retrospective studies demonstrate the effect of paternal age on various birth outcomes, including weight, premature deliveries, and various offspring abnormalities

[40, 41]. Additionally, recent research has begun to elucidate associations between aged fathers and increased incidence of obesity in offspring. These findings were independent of maternal age and other outside factors [42]. However, the etiology of the increased frequency of various disorders in the offspring of aged males remains poorly defined, though there are likely candidates.

In both sexes, aging alters DNA methylation marks in most somatic tissues throughout the body [43, 44]. Because of its prevalence in other cell types, aging-associated DNA methylation alteration is likely to occur in sperm as well. In fact, Oakes et al. have described age-associated hypermethylation at specific genomic loci in both sperm and liver tissue in male rats [44]. Similarly, our laboratory has identified increased global DNA methylation associated with age in human sperm from fertile donors (unpublished data). In further support of this idea is work demonstrating that frequently dividing cells have more striking methylation changes associated with age than do cells which divide less often [45]. Additionally, a recent study also indicates that, at specific gene promoters, there is increased DNA methylation in the offspring of older fathers [46]. These data further suggest the possibility of heritable DNA methylation alterations associated with advanced paternal age.

In addition to DNA methylation alterations there are data to suggest alterations in chromatin packing that occur with age as well. It has been suggested that chromatin remodeling plays a key role in cellular senescence, organismal aging, and age-associated disease and thus could play a role in age-associated sperm alterations that may ultimately affect the offspring [47]. In fact, Nijs et al. described altered chromatin packing associated with age as assessed by the sperm chromatin structure assay [48]. The subtle nature of the effect and, in some cases, the absence of well-characterized genetic factors, in addition to the aging-associated somatic cell methylation alterations, suggest that a major contributing factor to the increased prevalence of various diseases among the offspring of aged fathers is the sperm epigenome.

3.4 Reproductive Consequences of Age-Related Changes in Sperm

3.4.1 Fecundity

Among the consequences of delayed paternity, and likely the most dramatic alteration that occurs with increased paternal age, is that of decreased fecundity. Though very different from the universal and abrupt age-associated cessation of fertility seen in females, there is a significant decline in a male's capacity to produce viable offspring that is correlated with age. However, the age at which an individual male's reproductive capacity declines and even the frequency of this decline among a population of men remains controversial. Despite this, there are many studies that demonstrate an age effect on male fecundity with study groups, including natural conception, artificial insemination, and in vitro fertilization.

In an observational study performed in the United Kingdom in 2003, Hassan et al. found that men >45 years of age had a fivefold increase in their time to pregnancy in comparison to individuals <25 years of age [49]. Interestingly, when compared to males <25, men 45 and older were also 12.5 times more likely to have a time to pregnancy of greater than 2 years [49]. As expected, this effect is amplified when the female member of a couple is of advanced reproductive age as well (35–39). In these couples, men >40 were more than two times more likely to fail to conceive during a 12 month period in comparison to men <40 [50]. Additionally, when taken into account unsuccessful pregnancies in the same groups men over 40 were three times less likely to produce viable offspring than do the younger cohort [50]. Other studies support these data by suggesting an increased frequency of fetal loss to those fathered by older men, increased time to pregnancy, and decreased probability of conception [51–53]. However, there are conflicting data which suggest little to no effect of paternal age on fertility in natural conception [54].

Research has also described effects of paternal age on the outcomes of assisted reproductive techniques. A total of 17,000 intrauterine insemination

(IUI) cycles analyzed in a French study revealed that the pregnancy rate for couples whose male partner was less than 30 years of age had a pregnancy rate of 12.3 % where couples whose male partner was over 30 years of age had a significantly lower pregnancy rate of 9.3 % after adjusting for female age [55]. Similarly, in 1995, Mathieu et al. showed that increasing male age (≥35 years of age) was associated with decreased rates of conception [56]. However, these data are controversial. Additional studies have failed to find a paternal age effect on IUI pregnancy rates [57]. Other studies have analyzed the paternal age effect on in vitro fertilization (IVF) success with a similar controversy. Many studies suggested that there is a paternal age effect in achieving viable pregnancy outcomes in IVF cycles [58] and also have suggested that this effect is amplified with partners of advanced maternal age [59]. In large studies involving the use of donor eggs in an IVF cycle showed a significant effect of paternal age on pregnancy outcome [60]. However, an even more recent study that corrected for age of the egg donor found no effect of paternal age on pregnancy outcome [61].

3.4.2 Disease Risk in Offspring

As would be expected, the numerous genetic and epigenetic changes that occur to sperm through the aging process are associated with elevated risk of some diseases in the offspring of older fathers. These include several rare, autosomal disorders, disorders involving expanded trinucleotide repeats, offspring aneuploidy, certain cancers, and several neuropsychiatric disorders. These diseases and associated risks will be discussed below. While risks of these disorders are demonstrably elevated in offspring of older fathers, it is important to emphasize that the paternal age contribution to the increased risk is generally quite low (with the exception of the autosomal dominant and triplet repeat disorders) and absolute risk for any of these disorders remains quite low.

3.4.2.1 Autosomal Dominant Disorders

Rare autosomal disorders, including Apert syndrome and achondroplasia, are among the most striking and earliest characterized examples of increased disease risk as a consequence of advanced paternal age. As early as 1912, it was observed that sporadic cases of achondroplasia, a dominantly inherited form of dwarfism, was most often found in the last-born children of a family [29]. More recently, a number of other diseases have been shown to display similar paternal age effects.

A dozen diseases showing a significant paternal age effect were described in a paper more than three decades ago, and several others have been described since that time [62]. In addition to achondroplasia and Apert syndrome, the list of autosomal dominant disorders that display a paternal age effect includes acrodysostosis, fibrodysplasia ossificans progressive, neurofibromatosis, multiple endocrine neoplasia 2A (MEN 2A) and MEN 2B, and syndromes including Marfan, Treacher-Collins, Crouzon, Noonan, and Pfeiffer, among others [62].

Remarkably, many of these conditions, including Apert syndrome, achondroplasia, Crouzon syndrome, Pfeiffer syndrome, MEN 2A, and MEN 2B, involve mutations in three genes, *FGF3R*, *FGFR2*, and *RET* [29, 63]. Moreover, in almost every case where parental origin of the de novo, disease-causing mutation in these genes was assessed, the mutation was paternally derived [29, 63–68]. In addition, the mutated loci linked to many of these disorders are among the most frequently mutated nucleotides in the entire genome [29]. These observations led to the hypothesis of selfish spermatogonial selection, the idea that some spermatogonial mutations confer some advantage, leading to clonal expansion of mutant sperm over time [63, 69]. This mechanism may explain, at least in part, the molecular basis for the increased incidence of these disorders with advanced paternal age.

While it is well established that increasing paternal age does increase the risk for numerous autosomal dominant disorders, it is important to note that the absolute risk for these diseases remains quite low. Additional research is required to fully characterize the mechanisms involved in increased transmission of these diseases by older fathers.

3.4.2.2 Trinucleotide Repeat Disorders

In addition to the association between point mutations in the male germline and male age, there is also evidence to suggest that other genomic changes, namely changes in trinucleotide repeat length, are also more frequent in the germline of older men. The cause of Huntington's disease has been traced to an expanded block of CAG tandem repeats within the Huntingtin (*HTT*) gene [70]. Longer triplet repeats in *HTT* result in altered protein function and Huntington's symptoms. It was demonstrated that repeat expansion is almost entirely driven through the male germline [71], and the extent of repeat expansion is significantly associated with paternal age [72].

Myotonic dystrophy (DM) is another disease associated with trinucleotide repeat expansion. Like Huntington's disease, expanded CTG repeats are more frequently transmitted from the father [73], and paternal age appears to be a risk factor for transmission of the disease [74]. One large study of 3,419 cases of Down syndrome did find a significant paternal age effect after adjusting for maternal age when mothers were older than 35, and the paternal age effect was most significant when maternal age was over 40 [75].

3.4.2.3 Offspring Aneuploidy

The majority of aneuploidies are embryonic lethal, however trisomies 13, 18, and 21 along with sex chromosome aneuploidies (XXY, XYY, XXX, XO, etc.) are compatible with life. The great majority of somatic aneuploidies are maternally derived. For example in a cohort of 352 cases of Down syndrome, approximately 91 % were of maternal origin, and a maternal contribution to other cases of trisomy involving chromosomes 13, 14, 15, and 22 were similar, ranging from 83 to 89 % [76]. Interestingly, the story is different for sex chromosome aneuploidies, with a little more than half of cases being paternally derived [20].

Given the relatively minor effect of paternal age on sperm aneuploidy rates, it is not surprising that epidemiologic data for the paternal contribution to trisomic offspring generally do not support a paternal age effect [8, 77, 78]. A recent study based on 22 EUROCAT congenital anomaly registers identified a marginally significant association between paternal age and Klinefelter syndrome [79]. Several studies have evaluated the relationship between paternal age and incidence of Down syndrome, and in general have reported a weak paternal age effect [80] or no effect at all [81]. Based on available data, clearly the paternal age effect on offspring aneuploidy is relatively small and is eclipsed by the significant maternal age effect.

3.4.2.4 Cancer

Based on the current literature, it appears that paternal age may have an effect on incidence of various types of cancers in offspring. These data are intriguing but remain quite controversial. One of the most heavily studied classes of disease in these studies is hematological cancers. A recent epidemiological study has described a decreased risk of acute myeloid leukemia in firstborn children, indirectly suggesting that maternal and paternal age may play a role in the frequency of cancer incidence in the offspring. The same study was able to directly detect an increased risk of being diagnosed with any form of childhood leukemia in children sired by fathers of between 35 and 45 years of age when compared to fathers <25 years of age [82]. In agreement with these data is research by Murray et al. which suggests that children born to fathers >35 years of age are 50 % more likely (relative risk = 1.5) to receive a diagnosis of a childhood leukemia [83]. However, a Swedish epidemiological study published in 1999 detected no significant impact of paternal age on hematologic cancers [84].

The impact of paternal age on offspring cancer incidence is not limited to hematologic metastases. There also appears to be an increased risk of developing childhood central nervous system tumors in the offspring of older fathers. One retrospective study showed that children born to a father >30 years of age were at a 25 % increased risk of developing a childhood brain tumor compared to children of fathers <25 years [84]. Similarly, Yip et al. demonstrated that the offspring of fathers >40 had an increased relative risk (approximately 1.7) of developing a central nervous system cancer [85].

Advanced paternal age also appears to affect the incidence of adult onset cancers in offspring.

The incidence of breast cancer has been shown to increase in the daughters of fathers who are >40 compared to fathers <30 [86]. Similarly, prostate cancer risk increases by approximately 70 % in the offspring of fathers >38 years of age compared to the children of fathers <27 years of age [87].

The mechanism behind this effect is likely multifactorial and may additionally vary by race. However, there are some candidates that likely play at least some role in the etiology of increased incidence of multiple cancers seen in the offspring of aged fathers. Environmental exposures that accumulate throughout the life of a male are one of the most likely effectors, as this may affect subtle DNA mutations and epigenetic alterations that are capable of being inherited. In fact, as mentioned earlier, there are some data that suggest that the offspring of older fathers have increased levels of DNA methylation at specific loci [46]. If any of these alterations (gene mutations or epigenetic modifications) occur at tumor suppressor genes or other important genes in the etiology of various cancers, the result would be increased cancer incidence as is seen in the current literature. Though this correlation is intriguing, it should be noted that much work is still required to further define the effects of paternal aging on the incidence of cancer in offspring.

3.4.2.5 Neuropsychiatric Disorders

In recent years, with the application of genomic tools, the genetic complexity of neuropsychiatric disorders is becoming increasingly apparent. However, it has long been suggested that advanced paternal age is a risk factor for schizophrenia [88], and more recently, advanced paternal age has been implicated in risk for autism, bipolar disorder, behavioral disorders, and reduced cognitive ability.

The paternal age effects on schizophrenia risk have been widely studied [89–91]. A recent meta-analysis representing 24 qualifying studies confirmed advanced paternal age to be a significant risk factor for schizophrenia [89]. In this study, the authors reported a slight but significant increase in the risk of developing schizophrenia in offspring from fathers >30 years of age, with relative risk (RR) increasing in older fathers. At the extreme, a combined RR for schizophrenia in the offspring of fathers >50 years of age compared with fathers age 25–29 was 1.66 [89]. Interestingly, there also appears to be a slight but significant risk of schizophrenia in offspring of fathers < 25 years (RR = 1.08) only in male offspring [89].

Associations between paternal age and risk of autism spectrum disorders (ASD) have also been thoroughly investigated, with two meta-analyses confirming a significant association [92, 93]. In the most recent population-based study and meta-analysis, it was estimated that fathers >50 years of age had a 2.2-fold increased risk of autism in offspring compared with men aged 29 years or less [93].

The data regarding the association between paternal age and other neuropsychiatric and behavioral disorders are less clear, but there does seem to be an increase in bipolar disorder [94, 95] and behavioral issues [96, 97] in children of older fathers. In addition, some studies indicate that children of older fathers display slightly reduced IQ compared with children of younger fathers [98, 99], although the differences are small, and conflicting reports exist [100].

While evidence clearly suggests that paternal age does have some impact on neurological development and the incidence of neuropsychiatric disorders, the mechanisms for neurodevelopmental changes have not been elucidated. It has been suggested that increased risk may be related to increased mutations [101], changes in gene dosage as a result of copy number changes in the genome [102], or epigenetic changes associated with age [103]. It is also likely that behavioral factors in the fathers that result in delayed marriage also contribute [88], as these factors are very difficult to quantify and correct for in epidemiological studies.

3.4.3 Consequences in Context

From the available data, it is clear that advanced paternal age affects sperm quality, fecundity, and offspring health. However, this topic is only beginning to be thoroughly explored partially due to the recently growing trend of delayed parenthood that

appears to have driven increased media attention toward to the study of advanced paternal age and offspring health. This has placed many physicians in the difficult position of consulting concerned patients regarding their capacity to produce healthy offspring with only scant amounts of data from a field of study in its relative infancy. This discussion is fascinating and extremely complex as a result of the socioeconomic, emotional, and general health issues involved. Physicians should be prepared to address many questions from their patients, but should specifically be able to address two main concerns in this discussion. The first are patients who request to preemptively store sperm at relatively young ages as an alternative to natural conception at an advanced age. The second are male patients who seek advice on the "risks" of having children at advanced age. In either case, the patients must be well informed and comfortable in making their decisions.

Are cryopreserved sperm from a young healthy individual more capable of producing healthy offspring than fresh sperm from the same individual collected at an advanced age? This central question in the paternal aging debate is not easily addressed. In fact, the most accurate answer would be that we simply do not know. It is clear that advanced paternal age has been associated with increased incidence of many disease states in the offspring as has been previously outlined. It is also known that there is a slightly increased risk of birth defects in children conceived through in vitro fertilization (the advanced reproductive technology that would most likely be used in these cases). Additionally, though still controversial, it has been demonstrated that the cryopreservation of sperm, even in the presence of cryomedium, can result in DNA damage thus compounding the problem of using stored samples as an alternative to natural conception at an advanced age [104]. Despite this, because of the low risk in cryopreservation of male gametes, if a patient desires to store sperm at a young age with the intent of future use, it would not be unreasonable to support this decision if the patient has been well educated on the available data.

Patients of advanced age who are considering having children but have not previously stored sperm may also seek medical advice on whether or not they should attempt parenthood based on the recent data that demonstrates increased relative risk to the offspring. Would they be placing their offspring at a significant risk/disadvantage? In response to this question it is important to understand that while the data do suggest a relative increase in the risk of offspring of aged fathers developing many disorders and diseases including, but not limited to schizophrenia, autism and even cancer, the absolute risk of these are still very low. For example, the risk of developing childhood leukemia is approximately 1 in 25,000 in the general public, and in the offspring of older fathers that risk climbs to 1 in 17,000, approximately a 50 % increase [83]. Though the relative risk in this case is statistically significant, the absolute risk to the offspring of an aged father actually developing leukemia remains very low. It will be important for physicians to additionally encourage patients to consider their familial relationships and the emotional benefits of having children and weigh these with the subtle increases in risk of having children at an older age. In consulting male patients of an advanced age, the data do not support the recommendation of halting attempts at conception because of the risks to the offspring as it does in advanced maternal age. Despite this it is important to consider these risks and understand that the cumulative data on the disorders that have relatively increased prevalence in the offspring of older fathers may dissuade some from having children at an older age.

3.5 Conclusions

In recent years, we have learned a great deal regarding the effect of aging on male fertility. Advanced paternal age is negatively associated with many semen parameters, and these negative effects likely drive the general decrease in fertility and fecundity seen in males of advanced age. Though not an abrupt and complete loss of fertility as seen in advanced maternal aging, there is a gradual decrease in gamete quality associated with aging in males. This decrease in quality includes DNA damage, various genetic mutations,

and epigenetic alterations that appear to be capable of causing abnormalities in the offspring. Though we currently have evidence to support the paternal age associated increase in offspring disease susceptibility, the absolute risk remains quite low. Despite this, couples with an aged male partner should consider these risks and discuss them with their health care provider to determine their best course of action in their desire to conceive a child.

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CHAPTER 3

PATERNAL AGING AND ASSOCIATED INTRAINDIVIDUAL ALTERATIONS OF GLOBAL SPERM 5-METHYLCYTOSINE AND 5-HYDROXYMETHYLCYTOSINE LEVELS

The following chapter is a reprint of an article coauthored by myself and Aston KI, Cairns BR and Carrell DT. It was originally accepted and published on-line by *Fertility & Sterility*, May 2013 (copyright by American Society for Reproductive Medicine, Published by Elsevier Inc.). Reprinted with permission.

Paternal aging and associated intraindividual alterations of global sperm 5-methylcytosine and 5-hydroxymethylcytosine levels

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Objective: To evaluate the relative intraindividual changes in sperm 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) levels associated with age and to compare the levels of 5-hmC in mature human sperm to blood DNA.
Design: Prospective research study.
Setting: University-based andrology and in vitro fertilization (IVF) laboratory.
Patient(s): Fifteen known fertile sperm donors, 22 other known fertile controls, and 41 male blood donors from a general population tissue bank.
Intervention(s): None.
Main Outcome Measure(s): Measurements of global 5-mC and 5-hmC levels via an enzyme-linked immunosorbent assay (ELISA)-based assay.
Result(s): Global sperm 5-mC levels exhibit a statistically significant increase with age, and a similar trend was seen in 5-hmC levels. On average, in the age ranges we analyzed, 5-mC increased by 1.76% per year, and 5-hmC, though more variable, increased by approximately 5% per year. Additionally, we found that 5-hmC levels in sperm are 32.59% of that found in blood DNA.
Conclusion(s): Global sperm DNA methylation patterns are stable over short periods of time but increase with age, which raises important questions regarding the risks of advanced paternal age. Additionally, as we would predict for a transcriptionally quiescent cell type, 5-hmC levels are statistically significantly lower in human sperm than in blood DNA. (Fertil Steril® 2013;100:945–51. ©2013 by American Society for Reproductive Medicine.)
Key Words: Advanced paternal age, global DNA methylation, 5-hydroxymethylcytosine, 5-methylcytosine, sperm DNA methylation

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Aging is known to severely affect female fertility, including elevating the risk of spontaneous abortion, chromosomal defects in the offspring, preterm delivery, and intrauterine growth restriction (1). In contrast, the effect of advanced paternal age on fertility, pregnancy outcome, and offspring health has received far less attention. However, recent studies linking older fathers to an increased prevalence of several neuropsychiatric disorders in the offspring has increased interest on the effect of advanced paternal age on offspring health. Specifically, various reports have linked increased paternal age at conception with bipolar disorder, schizophrenia, and autism in humans as well as decreased social and exploratory behavior in animal models (2–5). Additionally, studies have shown an association between advanced paternal age and increased DNA damage and decreased chromatin integrity in addition to adverse birth outcomes, including alterations in weight, premature delivery, and various other offspring abnormalities (6–9).

The etiology of the increased frequency of these various disorders in the offspring of aged males remains poorly defined, though epigenetic mechanisms are an obvious possibility. It has been demonstrated that DNA

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methyltransferase 3a (DNMT3a) and 3b (DNMT3b) expression increases with the age of the tissues investigated (10). Additionally, it is known that DNA methylation is altered in many somatic cell types with age, but similar changes in the sperm remain uncharacterized (11, 12). Despite this, the age-related DNA methylation modifications that occur in virtually every other tissue in the body suggest that similar patterns exist in sperm. In further support of this idea is work demonstrating that frequently dividing cells have more striking methylation changes associated with age than do cells that divide less often (13). Thus, alterations in sperm DNA methylation represent an undefined but highly plausible mechanism contributing to the increased incidence of neuropsychiatric disorders seen in the offspring of older fathers.

Our understanding of the paternal epigenetic landscape has increased dramatically in the recent past. Among the important epigenetic marks in sperm is DNA methylation at cytosine residues (5-mC). These marks are typically associated with gene silencing by inhibiting access of transcription-related binding proteins to the DNA. Recently, there has been increased interest in the role of DNA demethylation intermediates such as 5-hydroxymethylcytosine (5-hmC), which is formed by the 10-11 translocase (TET) family of enzymes. These marks are thought to play a regulatory role in gene expression and/or poising. The current literature describes unique epigenetic marks in sperm that are capable of facilitating proper function in the mature gamete as well as marks that are uniquely capable of poising specific genes in the early embryo for activation (14, 15). These data establish the importance of the paternal germline for embryogenesis in a way that was never previously understood.

It is clear from the available evidence that the inheritance of DNA methylation alterations is a plausible candidate for the etiology of many heritable disorders previously considered idiopathic in nature. Additionally, aberrations in the sperm methylome may contribute to the elevated risk of certain disorders associated with advanced paternal age. We describe the relative changes in global DNA methylation that occur through the process of aging by comparing semen samples collected by sperm donors many years apart. This study involved three basic aspects. First, we sought to determine the effect of aging on sperm 5-mC and 5-hmC levels. Second, we analyzed the intra-individual variability of 5-mC and 5-hmC levels in samples collected within 14 days of each other. Finally, we evaluated the difference in 5-hmC levels in sperm and blood DNA.

MATERIALS AND METHODS

Study Participants

Our laboratory has a research tissue bank that includes sperm and blood samples from previous sperm donors. Fifteen semen donors of known fertility who had previously banked samples at our laboratory were asked to return and collect a sample in 2008 under a protocol approved by the institutional review board. The time between the sample collections varied between 9 and 21 years. The basic semen analysis data for the two collections are available in Supplemental Table 1 (available online). The age at early collection for the donors in our sample set was between 27 and 56 years (Fig. 1; Table 1). To determine the intraindividual variability of sperm DNA methylation levels, 17 sperm donors collected three samples during a period of less than 14 days. These samples were used to test the consistency of sperm DNA methylation over a very short period of time.

To compare the levels of 5-hmC in sperm and blood DNA, we analyzed 52 sperm samples and 41 blood samples from our general population tissue bank. These donors are from a nonselected pool of men (Table 1).

FIGURE 1

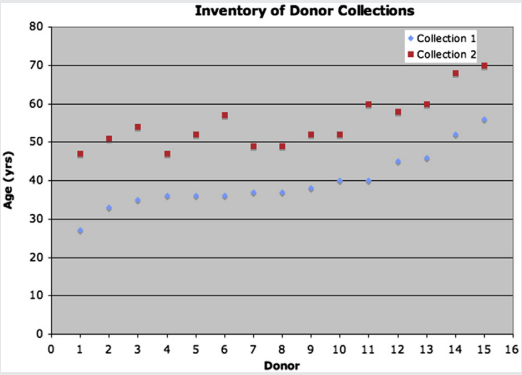

Illustration of the ages at which donors collected their samples. Nine of the 15 donors collected three samples, and the remaining six donors only collected two samples.

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Sample Collection and DNA Extraction

The men provided both semen and blood samples. The donors were required to strictly follow the University of Utah Andrology Laboratory collection instructions, which include abstinence time of between 2 and 5 days. After collection, the samples were frozen with test yolk buffer (TYB; Irvine Scientific) and were stored in liquid nitrogen before DNA isolation. No sperm selection method was employed as it was our aim to observe changes to all sperm in the ejaculate.

The samples were thawed and DNA extracted simultaneously to decrease batch effects. Sperm DNA was extracted using a sperm-specific extraction protocol used routinely in our laboratory. Before DNA extraction, we employed a

TABLE 1

Patient demographics of semen and blood donors.

Sample set	No. of patients	Body mass index (±SEM)	Age (±SEM)
Young	15	27.05 (±0.93)	39.33 (±2.02)
Aged	15	28.32 (±1.37)	53.27 (±1.92)
General population			
Blood samples	41	27.16 (±0.88)	37.64 (±1.65)
Sperm samples	52	26.44 (±0.46)	38.20 (±1.18)

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somatic cell lysis protocol that includes incubation in somatic cell lysis buffer (0.1% SDS, 0.5% Triton X-100 in DEPC H_2O) for 20 minutes on ice to eliminate white blood cell contamination. After somatic cell lysis, we performed a visual inspection of each sample to ensure the absence of all potentially contaminating cells before proceeding (16). Sperm DNA was then extracted using enzymatic and detergent-based lysis followed by treatment with RNase and finally by DNA precipitation using isopropanol and salt, with subsequent DNA cleanup using ethanol.

Blood samples were obtained by venipuncture, and they were immediately frozen and stored at −80°C until DNA extraction. Blood DNA was extracted using Puregene DNA purification kit (Gentra Systems) according to the manufacturer's recommendations.

Global 5-mC and 5-hmC Measurement

We used MethylFlash Colorimetric Methylated DNA Quantification Kit and HydroxyMethylated DNA Quantification Kit (Epigentek) for the quantification of 5-mC and 5-hmC, respectively. This enzyme-linked immunosorbent assay (ELISA) kit has been established as a quick, effective, and inexpensive method for the relative quantification of global 5-mC and 5-hmC levels in many tissues types from various species (17–20). The assays were performed according to the manufacturer's recommendations. Briefly, 100 or 200 ng of genomic DNA was used for 5-mC or 5-hmC quantification, respectively. It is important to note that the paired samples were analyzed simultaneously on the same plate in neighboring positions to decrease the possibility of experimental variance, and each sample was run in duplicate. Additionally we performed two technical replicates for 5-mC and 5-hmC assays to evaluate assay variability.

After incubation with the input DNA, the wells were washed, and a capture antibody was applied to each well, after which the wells were again washed and detection antibody applied. Use of enhancer solution and development solution created a color change proportional to the quantity of 5-mC or 5-hmC, and the samples were read on an automated plate reader at 450 nm absorbance. The use of a standard curve enabled the quantification of 5-mC or 5-hmC based on absorbance measurements.

Microarray Analysis

Paired samples from two representative donors were selected to confirm the global sperm methylation results that we observed. Extracted sperm DNA was bisulfite converted with EZ-96 DNA Methylation-Gold kit (Zymo Research) according to the manufacturer's recommendations. Converted DNA was then delivered to the University of Utah Genomics Core Facility and hybridized to Infinium Human Methylation 450 Bead Chip micro-array (Illumina) and analyzed according to Illumina protocols. Once the DNA were scanned and analyzed for quantities of methylation or lack of methylation at each CpG, we generated β value by applying the average methylated and unmethylated intensities at each CpG using the calculation: β value = Methylated/(Methylated + Unmethylated). This β value ranges from 0 to 1 and indicates the relative levels of methylation at each CpG, with highly methylated sites scoring close to 1 and unmethylated sites scoring close to 0.

Statistical Analysis

The relationship between age and 5-mC or 5-hmC quantification was measured in two ways. First, linear regression analysis was used to determine the relationship between age at time of collection and the levels of 5-mC and 5-hmC. Each of the 15 donors is represented twice in this data set (once for each collection). Second, to further investigate the variation that occurs within each individual with age, we normalized the 5-mC and 5-hmC levels for three collections from each individual. We accomplished this by determining the average 5-mC and 5-hmC levels that were found in each individual across three collections and then divided the 5-mC and 5-hmC levels at each collection by the average quantity for that individual. In this way, we were able to generate a normalized data point for each collection from each individual to assess the relative changes between collections for that individual.

Normalized data were subjected to Levene's homogeneity of variance analysis to determine whether DNA methylation in an individual over many years was more variable than over only a short period of time (i.e., 2 weeks). Finally, to determine the statistical significance of the difference in blood and sperm 5-hmC levels, we used a Student's t test. All statistical analyses were performed using the STATA 11 software package, and $P<.05$ was considered statistically significant.

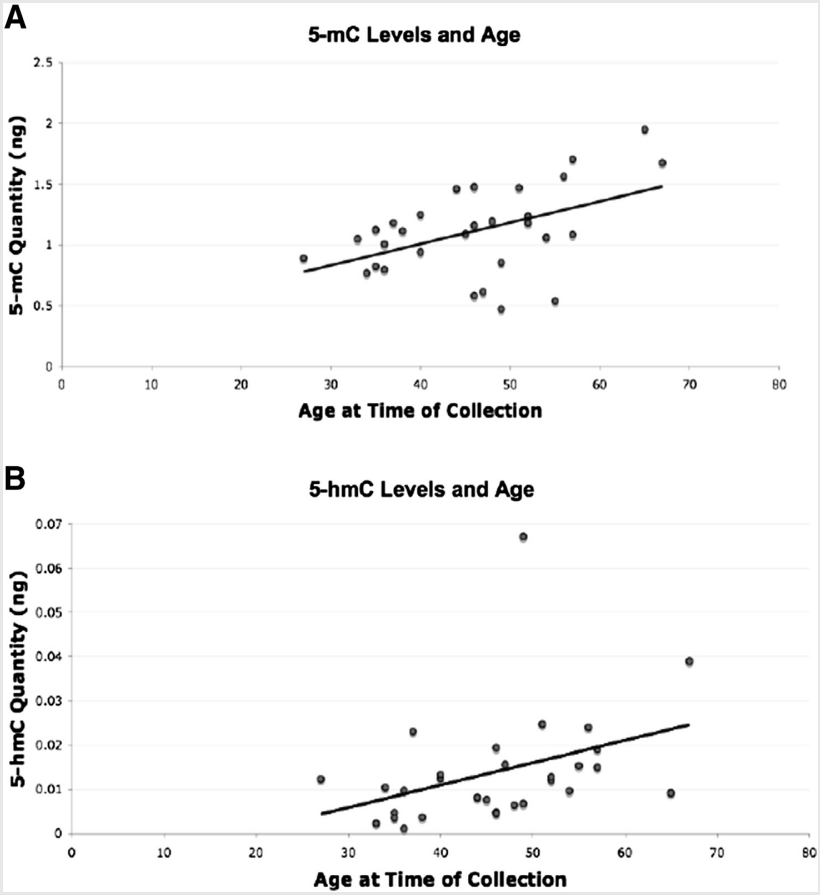
RESULTS

Aging and Sperm 5-mC and 5-hmC Levels

To evaluate the global DNA methylation alterations that occur with age, we analyzed the earliest available collection and the 2008 collection for each of the 15 donors (collection 1 and 2 in Fig. 1). The ages represented in this analysis ranged from 27 to 70 years. In our analysis, we found that there was a statistically significant positive correlation between paternal age and sperm global 5-mC levels ($r = 0.475$; $P=.008$) (Fig. 2A). A similar pattern was found with 5-hmC ($r = 0.38$; $P=.038$) (Fig. 2B). On average, 5-mC increased by 1.76% per year in our sample set; 5-hmC, though more variable, increased by approximately 5% per year in our sample set. It is important to note that, based on the general appearance of the linear regression plot, it is possible that within the 5-mC data set a multiphasic correlation exists to age such that 5-mC is relatively stable up to approximately 55 to 60 years of age, at which point 5-mC dramatically increases. However, we are unable to statistically confirm this relationship due to our low sample size in this age category.

To confirm these results, we additionally subjected paired samples from two representative donors to array analysis. The results demonstrated a modest increase in methylation with age in each sample at the ~450,000 CpGs probed, the majority of which are located in well-defined CpG islands and other

FIGURE 2



(A) Global sperm 5-methylcytosine (5-mC) is positively correlated with age based on linear regression analysis (R = 0.475; *P*=.008). (B) Similarly, it was found that 5-hydroxymethylcytosine (5-hmC) levels in sperm are positively correlated with age (R = 0.38; *P*=.038).

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regulatory regions. It is interesting that at the ~50,000 CpGs probed in regions outside of CpG islands, the CpG shores, CpG shelves, or other defined regulatory features, there was a 1.02% increase in average β value over a period of 15 years in the first donor and a 1.07% increase in average β value over a period of 10 years in the second donor (Supplemental Fig. 1). In both donors, this age-associated hypermethylation in regions outside of well-defined regulatory features was strongly statistically significant based on a paired *t* test ($P<.0001$).

Intraindividual Variability

To test the intraindividual variability associated with aging, samples from each donor were normalized to demonstrate the relative variability between samples, as described earlier. Two tests were performed: the first analyzed the variability that arose between the young and aged samples form the 15 donors over a period of 9 to 21 years, and the second analyzed intraindividual variability between ejaculates collected within 14 days of each other.

We found that the 5-mC levels in samples collected within a short interval had a relatively low degree of intraindividual variance with a coefficient of variance (CV) of 12.42% (standard error of the mean [SEM] ± 0.0181). Samples collected between 9 and 21 years apart had a CV of 32.23% (SEM ± 0.0551). We subjected these two sample sets to a homogeneity of variance statistical analysis (Levene's test) and determined that samples collected years apart showed a statistically significantly increased degree of variance ($P<.001$). The same analysis was performed for 5-hmC levels, and we found a high degree of intraindividual variance in samples collected years apart (CV = 48.69%; SEM ± 0.0904). It is interesting that in samples collected within a short interval we found similar variability (CV = 43.36%; SEM ± 0.0834). These results were not statistically significantly different ($P=.587$). Additionally, we ran two sets of

technical replicates for both the 5-mC assay and the 5-hmC assay. The CV for the 5-mC assay for both replicates was 6.8% and 6.3%. For the 5-hmC assay, this variability was slightly higher with a CV of 12.9% and 13.2%.

Sperm Versus Blood DNA 5-hmC Content

While measuring the relative levels of 5-hmC in sperm samples we noticed that nearly all samples were at approximately the lowest threshold of the assay. In fact, in multiple samples 5-hmC was undetectable. With this in mind, we sought to investigate sperm 5-hmC levels relative to blood levels to confirm our results and provide a baseline measure from another tissue type known to have very low 5-hmC levels (21). Based on a *t* test analysis of 41 blood samples and 52 sperm samples, we found a statistically significantly decreased level of 5-hmC in sperm DNA compared with blood ($P<.0001$) (Fig. 3). We found the difference to be quite striking, with sperm DNA having approximately only 32.59% the amount of global 5-hmC of blood DNA.

DISCUSSION

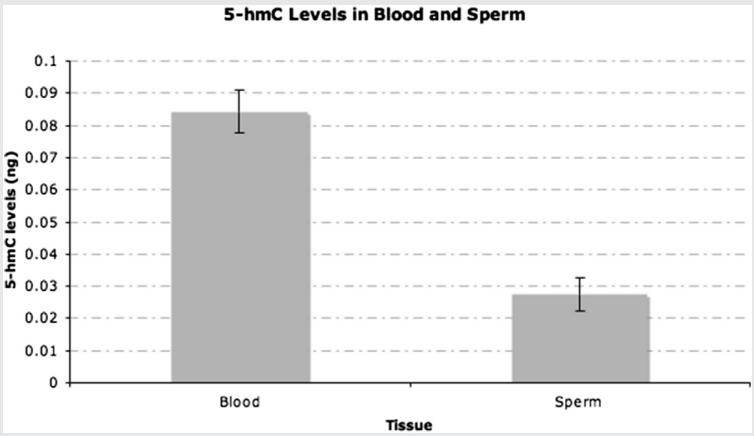
Our data indicate a statistically significant increase in global sperm 5-mC levels associated with age in a known fertile population. This difference was coupled with increased intraindividual variance in global sperm 5-mC associated with age when compared with the variability seen between collections only a few days apart. These data demonstrate that sperm DNA methylation levels undergo statistically significant changes with age, although the impact of these alterations is unknown.

Our goal was to perform a descriptive study with a unique sample set to analyze the epigenetic alterations in sperm that are associated with age, as these are likely candidates to contribute to the etiology of some cases of paternal age related disorders. Because this is a descriptive study, it will be important in the future to directly study the impact these alterations have on offspring in animal models and largely expanded human studies. In light of the modest number of donors that we were able to analyze in this study, as a result of the difficult nature of obtaining these paired samples from collections greater than 10 years apart, we did not investigate any adverse outcomes from any of the donor offspring as the sample size was too small to detect any change in frequency of offspring abnormalities. Despite this, our findings, specifically DNA methylation alterations with age, are in agreement with many other studies that have previously reported alterations to DNA methylation marks and DNMT expression in various somatic tissues (10–12).

In light of the degree of global alterations to the sperm methylome identified in this study, and as our preliminary array data suggest, it is likely that a large portion of these changes have occurred outside of CpG islands or other regulatory regions. Thus, these changes may be occurring at repeatable elements or other noncoding, nonregulatory regions, which account for the majority of methylation in the genome. Based on our current understanding of the sperm epigenome, DNA methylation alterations in these outlying regions may be limited in their ability to produce biologically relevant changes. However, these large-scale, global alterations to the epigenetic program may also reflect more subtle and important changes, such as alterations to gene promoter methylation. Additionally, it should be noted that the antibody used to detect 5-mC is incapable of discriminating between CpG methylation and non-CpG methylation, which could also contribute to the changes in global methylation status reported herein.

In addition, recent literature has indicated that there is an association between human sperm global hypermethylation and apoptosis, suggesting that our findings may be a result of the increased incidence of apoptotic cells in older males'

FIGURE 3



Forty-one blood samples from our general population tissue bank were analyzed for 5-hydroxymethylcytosine (5-hmC) levels. Fifty-two sperm samples from donors and known fertile controls within our tissue bank were also assessed for 5-hmC levels. We determined that sperm 5-hmC levels are statistically significantly lower than the levels found in blood ($P<.0001$).

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sperm population (22). Though there are many potential explanations for the alterations we have seen with age, it is impossible to determine the biologic relevance of these changes without further investigation with higher resolution technologies, such as bisulfite sequencing or expanded microarray analysis. Despite this, we have described an intriguing alteration to the sperm epigenome associated with age in a unique sample set. Although they are not descriptive of the precise CpG loci that have been altered or independently explanatory of any mechanism for these changes, these data warrant further investigation as they provide us with the first available characterization of sperm DNA methylation alterations associated with age.

In addition to the changes in 5-mC content in sperm cells, we found alterations to the levels of 5-hmC with age. Specifically, we found that global sperm 5-hmC levels increase with age. It should be noted that while we did observe a statistically significant increase in 5-hmC associated with aging and a high degree of variability (CV = 48.69%) in samples collected between 9 and 21 years apart, the samples collected only a few days apart displayed a statistically similar level of variability (CV = 43.36%). With this in mind, it will be important to further investigate these observed alterations with higher resolution technologies in the future.

The low levels of 5-hmC in sperm and the lack of literature describing this epigenetic modification in the male gamete led us to investigate this unique finding further. Previous studies have demonstrated that human blood has among the lowest levels of 5-hmC among tissues analyzed, thus we selected blood as a reference point to determine the relative differences in global 5-hmC levels (21). Though the idea is still controversial, it has been hypothesized that gene body 5-hmC enrichment is associated with transcription activation (21, 23). If correct, this proposed mechanism would suggest that 5-hmC levels should be very low in the transcriptionally quiescent mature sperm. In agreement with this hypothesis, and despite the high degree of variability seen between sperm samples, we found a strikingly statistically significant decrease (approximately threefold) in 5-hmC levels in sperm when compared with blood DNA. These findings also comport with the relatively high levels of global 5-hmC that have been reported in whole testis tissue (21), where somatic cells and spermatogonial stem cells are transcriptionally active.

Our findings regarding 5-mC alterations in human sperm associated with age represent the first study of its kind. Similarly, from our investigation, these data represent the first analysis of global 5-hmC levels in human sperm. These unique data provide an excellent foundation upon which future studies can expand these findings with the use of high-resolution technologies to enable us to observe changes at the single CpG level across multiple loci. This future work will allow us to understand not only the loci at which alterations occur but to predict their biologic relevance to the mature sperm, the embryo, and possibly the offspring. Additionally, our data demonstrating low levels of 5-hmC in the mature sperm provide evidence that supports the previously proposed hypothesis that 5-hmC enrichment in gene bodies is associated with transcriptional activity. Taken together, these data describe relative changes to sperm DNA methylation associated with age as well as the differences in 5-hmC levels in sperm versus leukocytes. Future studies should further investigate these changes to elucidate the possible effects these alterations may have on offspring phenotype.

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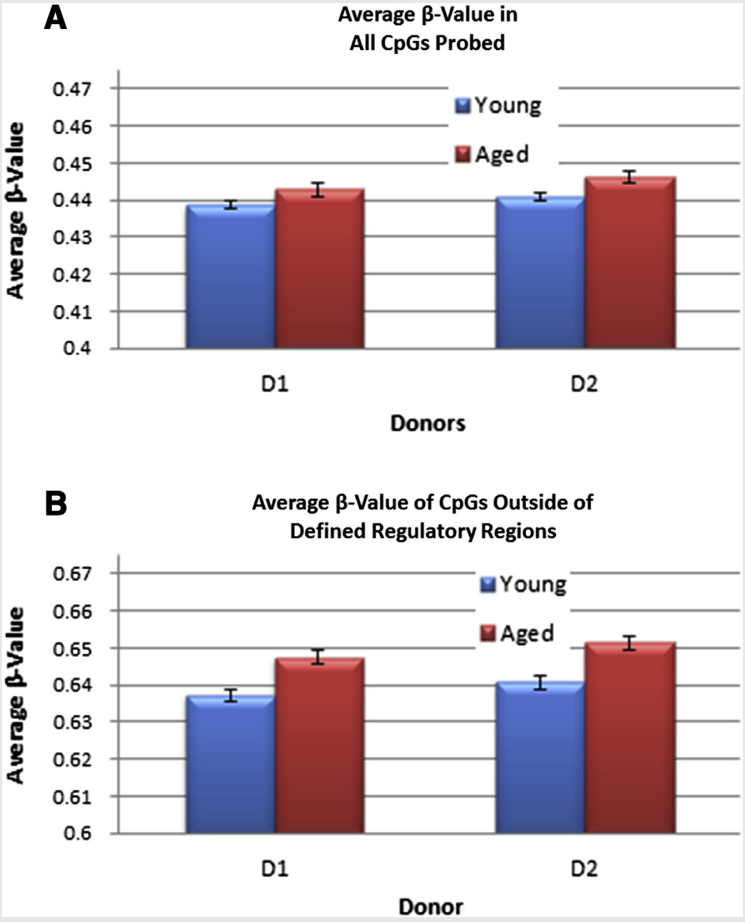
SUPPLEMENTAL FIGURE 1



Microarray analysis of paired samples from two representative donors from our study population. Average β values for all 450,000 CpGs probed on the array (A) and for the ~50,000 CpGs that are located outside of defined regulatory regions (B) are represented here. In both donors, the increase in methylation at CpGs outside of defined regulatory regions was strongly statistically significant based on a paired t test.

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SUPPLEMENTAL TABLE 1

Donor semen analysis data.

Parameter	Young (±SEM)	Aged (±SEM)	*P* value
Volume	3.78 (±0.46)	2.85 (±0.45)	.0142
Million/mL	125.4 (±9.16)	145.56 (±15.57)	>.05
Total count	434.32 (±53.67)	424.67 (±88.69)	>.05
Total motile	63.38 (±1.64)	61.25 (±4.34)	>.05
Percentage live	69.08 (±1.47)	61.0 (±3.93)	>.05

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CHAPTER 4

AGE-ASSOCIATED SPERM DNA METHYLATION ALTERATIONS: POSSIBLE IMPLICATIONS IN OFFSPRING DISEASE SUSCEPTIBILITY

Introduction

The effects of advanced paternal age have only recently become of interest to the scientific community as a whole. This interest has likely arisen as a result of recent studies that suggest an association with increased incidence of diseases and abnormalities in the offspring of older fathers. Specifically, offspring sired by aged fathers have been shown to have an increased incidence of neuropsychiatric disorders (autism, bipolar disorder, schizophrenia, etc.) [1-3], trinucleotide repeat associated diseases (myotonic dystrophy, spinocerebellar atixia, Huntington's disease, etc.) [4-7], as well as some forms of cancer [8-11]. Though these are intriguing data, we know very little about the etiology of the increased frequency of diseases in the offspring of older fathers. Among the most likely contributing factors to this phenomenon are epigenetic alterations in the sperm that can be passed on to the offspring.

These studies are in striking contrast to the previously held dogma that the mature sperm are capable only of the safe delivery of the paternal DNA and little more. Intriguingly, with increased investigation has come mounting evidence that the sperm epigenome is not only well suited to facilitate mature gamete function but is also

competent to contribute to events in embryonic development. It has been established that even through the dramatic nuclear protein remodeling that occurs in the developing sperm, involving the replacement of histone proteins with protamines, some nucleosomes are retained [12]. Importantly, histones are retained at important genomic loci for development, suggesting that the sperm epigenome is well suited to poise the paternal DNA for embryogenesis. Similarly, DNA methylation marks in the sperm have been identified that likely contribute to embryonic development as well [13]. These data strongly support the hypothesis that the sperm epigenome is not only well suited to facilitate mature sperm function, but that it also contributes to events beyond fertilization.

The contribution of the sperm appears to reach beyond embryogenesis as well. The remarkable claim that sperm, independent of gene mutation, may be capable of affecting phenotype in the offspring was initially proposed as a result of large retrospective epidemiological studies observing changes in the frequency of diseases in the offspring of fathers who were exposed to famine conditions in the early 19th century [14,15]. Recently, many studies utilizing animal models have discovered similar patterns that comport with the epidemiological data. Specifically, in male animals fed a low protein diet, offspring display altered cholesterol metabolism in hepatic tissue [16]. However, the etiology of this phenomenon is poorly understood. Despite this, there are multiple likely candidates that may drive these effects, such as DNA methylation.

Methylation marks at cytosine residues, typically found at cytosine phosphate guanine dinucleotides (CpGs), in the DNA are capable of regulatory control over gene activation or silencing and are additionally believed to help prevent alternative transcription start sites. These roles are dependent on location relative to gene

architecture (promoter, exon, intron, etc.). Because these heritable marks are capable of driving changes that may affect phenotype, they represent a logical candidate for a mechanism to explain the increased disease susceptibility in the offspring of older fathers. In this study, we have analyzed the age-associated sperm DNA methylation alterations that are common among the individuals in our study population to determine the magnitude of sperm DNA methylation changes over time and whether specific regions are consistently altered with age.

Results

Descriptive Analysis

Our results indicate that, in general, sperm DNA methylation marks are robust within individuals as they age, though there are alterations that occur. Based on our pyrosequencing analysis of long interspersed elements (LINE), global sperm DNA is significantly hypermethylated with age as previous data from our lab suggest (Figure 4.1.) [17]. In addition to this global change, we identified multiple regions of age-associated methylation alterations. We have identified intraindividual regional methylation alterations between paired samples (young and aged) that consistently occur within the same genomic windows in most or all of the donors screened. Remarkably, these alterations occur whether the individual collected the samples in their 20s and 30s or in their 50s and 60s. Using a sliding window analysis, coupled with regression analysis as an additional filter, we identified a total of 139 regions that are significantly hypomethylated with age (Log2ratio $\leq$-0.2) and 8 regions that are significantly hypermethylated with age (Log2ratio $\geq$0.2; Table 4.1).

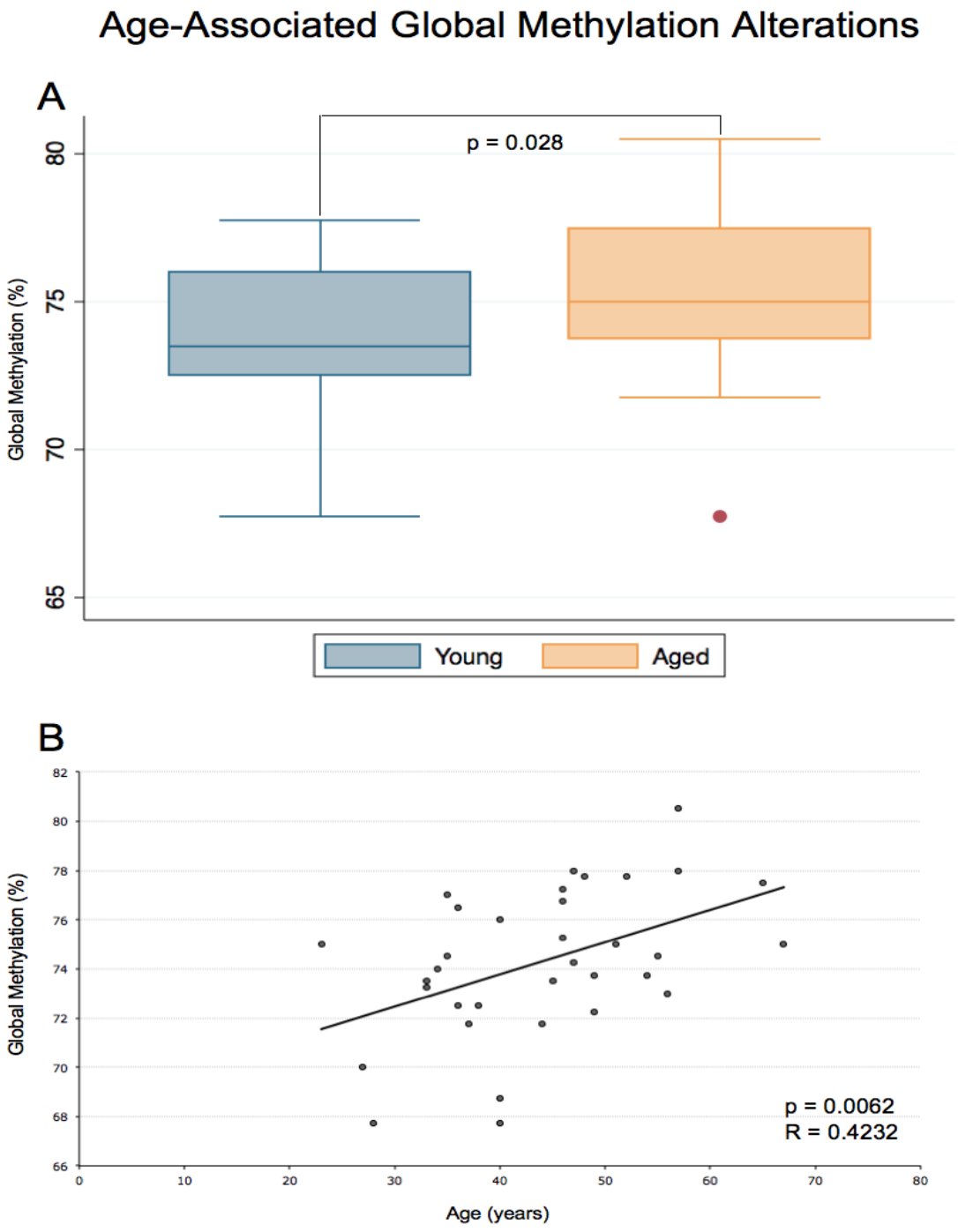

Figure 4.1: Pyrosequencing results for the LINE-1 global methyaltion assay. The box plot depicts significantly increased average global methylation with age in paired samples from all 17 donors based on a two tailed t-test (p=0.028; A). Global methylation was also stratified based only on age at the time of collection for each sample from all 17 donors (a total of 34 samples with each donor represented twice). Linear regression analysis confirmed the significant increases in global sperm DNA methylation with age (p=0.0062; B).

Table 4.1: Genomic Features of Significantly Altered Windows

Gene	Genomic Feature	CpG Island Association	DMR	Log2 Ratio	FDR	R^2
ARC	Gene Body	North Shore	N/A	-0.2433	65.69	0.1902
ATHL1	Gene Body	Island/South Shore	N/A	-0.2932	65.69	0.1714
ATN1	Promoter	North Shelf	N/A	-0.3702	65.69	0.4421
ATXN7L3	Promoter	North Shore	N/A	-0.2158	65.69	0.3413
BEGAIN	Promoter	South Shore	N/A	-0.2747	65.69	0.4085
BLCAP	Gene Body	North Shore	N/A	-0.2366	65.69	0.4881
C1orf122	Promoter	North Shore	N/A	-0.2272	65.69	0.5157
C6orf48	Gene Body	South Shore	N/A	-0.2061	65.69	0.1544
CCDC114	Promoter	North Shore	N/A	-0.3703	65.69	0.5512
CCDC144NL	Promoter / Gene Body	Island	N/A	0.2034	65.69	0.1989
CFD	Promoter	North Shore	N/A	-0.2795	65.69	0.3099
CLIC1	Gene Body	South Shore	N/A	-0.2159	65.69	0.2098
CNN1	Promoter / Gene Body	N/A	N/A	-0.2591	65.69	0.2501
CNTNAP1	Promoter	North Shore	RDMR	-0.2157	65.69	0.3904
DLL1	Gene Body	Island/North Shore	N/A	-0.2937	65.69	0.1544
DOK1	Promoter	North Shore	CDMR	-0.2528	65.69	0.4926
DRD4	Gene Body	Island	N/A	-0.5705	65.69	0.3172
EFCAB4A	Gene Body	Island	N/A	-0.3166	65.69	0.2888
ELANE	Promoter / Gene Body	North Shore	N/A	-0.5163	65.69	0.1359
GAPDH	Promoter	North shore	RDMR	-0.2191	65.69	0.2135
GET4	Promoter	Island/North Shore	N/A	-0.2080	65.69	0.316
GPANK1	Gene Body	North Shore	RDMR	-0.2451	65.69	0.3234
GPR45	Promoter / Gene Body	Island/North Shore	N/A	-0.2399	65.69	0.3908
KCNF1	Gene Body	Island	N/A	-0.3344	65.69	0.1838
KCNQ1	Gene Body	Island/North Shore	N/A	-0.2991	65.69	0.2046
LOC154449	Promoter	North Shelf	N/A	-0.2064	65.69	0.122
MIR22HG	Gene Body	North Shore	N/A	-0.2347	65.69	0.2404
MPPED1	Gene Body	Island	N/A	-0.2851	65.69	0.1553
N/A	N/A	HMM Island	N/A	-0.2041	65.69	0.2629

Table 4.1: Continued

Gene	Genomic Feature	CpG Island Association	DMR	Log2 Ratio	FDR	R^2
N/A	N/A	Island/North Shore	N/A	-0.2363	65.69	0.3355
N/A	N/A	North Shore	N/A	-0.3082	65.69	0.2066
N/A	N/A	Island/North Shore	N/A	-0.3820	65.69	0.1795
PCOLCE	Promoter / Gene Body	North Shore	N/A	-0.2438	65.69	0.1543
PITPNM1	Promoter	North Shore	N/A	-0.2669	65.69	0.4935
PPP1R18	Gene Body	Island/North Shore	N/A	-0.2754	65.69	0.3867
PRSS22	Promoter	South Shore	N/A	-0.2486	65.69	0.5034
PYY2	Promoter / Gene Body	North Shore	N/A	-0.3241	65.69	0.6317
SECTM1	Gene Body	Island	N/A	-0.2568	65.69	0.3782
SYNE4	Promoter	North Shore	N/A	-0.2383	65.69	0.5805
TBKBP1	Gene Body	Island	N/A	-0.2449	65.69	0.4863
THBS3	Promoter / Gene Body	North Shore	N/A	-0.2657	65.69	0.5953
TNXB	Gene Body	Island	N/A	-0.3319	65.69	0.2436
UTS2R	Promoter / Gene Body	Island/North Shore	N/A	-0.2767	65.69	0.2616
ZNF358	Promoter / Gene Body	Island/North Shore	N/A	-0.2473	65.69	0.1876
KDM2B	Promoter	South Shore	RDMR	-0.3003	65.67	0.241
NSG1	Promoter	North Shore	N/A	-0.2899	65.47	0.5232
SEZ6	Gene Body	Island/North Shore	N/A	-0.4530	65.05	0.43
LMO3	Promoter	N/A	N/A	-0.3627	64.24	0.2074
HOXA10	Promoter / Gene Body	Island/North Shore	N/A	-0.2148	64.21	0.3354
DAPK3	Promoter	North Shore	RDMR	-0.3932	63.18	0.3728
N/A	N/A	Island/North Shore	N/A	-0.3281	62.21	0.2824
N/A	N/A	South Shore	N/A	-0.2993	62.03	0.125
NSMF	Gene Body	Island/North Shore	N/A	-0.2249	61.30	0.329

Table 4.1: Continued

Gene	Genomic Feature	CpG Island Association	DMR	Log2 Ratio	FDR	R^2
TOR4A	Promoter	Island/North Shore	N/A	-0.3046	61.09	0.3998
LDLRAD4	Promoter	N/A	N/A	-0.2502	60.61	0.264
N/A	N/A	North Shore	RDMR	-0.2866	58.83	0.5618
PTPRN2_3	Gene Body	North Shore	N/A	-0.2391	58.31	0.151
SSTR5	Gene Body	Island/North Shore	N/A	-0.2381	57.88	0.1457
LOC134368	Gene Body	South Shore	RDMR	-0.2695	57.71	0.292
GRB7	Promoter	N/A	N/A	-0.2087	57.48	0.3144
GNB2	Gene Body	South Shore	N/A	-0.2238	57.45	0.1312
SNHG1	Promoter	North Shore	N/A	-0.2004	57.39	0.404
LOC653566	Promoter	N/A	N/A	-0.2929	56.31	0.2672
N/A	N/A	HMM Island	N/A	-0.2479	56.06	0.1969
PDE4C	Gene Body	Island/South Shore	N/A	-0.2858	55.53	0.4673
DLGAP2	Gene Body	Island/North Shore	N/A	-0.2109	55.49	0.1296
MRPL36	Gene Body	North Shore	N/A	-0.2268	55.34	0.3998
NCOR2	N/A	HMM Island	N/A	-0.2106	55.34	0.583
N/A	N/A	North Shore	CDMR	-0.2107	54.57	0.1157
N/A	N/A	N/A	CDMR	-0.2813	52.81	0.2763
KCNA7	Promoter	South Shore	N/A	-0.3664	52.24	0.5066
CACNA1H	Gene Body	South Shore	N/A	-0.2855	51.96	0.1695
IRS4	Gene Body	North Shore	RDMR CDMR	-0.2273	51.23	0.2364
KRT19	Promoter	South Shore	N/A	-0.2701	51.08	0.3463
LRFN2	Gene Body	North Shore	RDMR	-0.2525	51.08	0.2967
WFDC1	Gene Body	Island	N/A	-0.2966	50.49	0.2675
APBA2	Promoter	N/A	N/A	-0.3989	50.10	0.3216
USP36	Gene Body	North Shore	RDMR	-0.3108	49.92	0.2693
PAX2	Gene Body	South Shore	N/A	-0.3545	49.15	0.2825
PTPRN2_1	Gene Body	North Shore	N/A	-0.2828	48.41	0.3052
N/A	N/A	North Shore	RDMR	-0.2138	47.98	0.4739
N/A	N/A	HMM Island	N/A	-0.2144	47.75	0.2672
UNKL	Promoter / Gene Body	Island/North Shore	N/A	-0.2483	47.55	0.4327

Table 4.1: Continued

Gene	Genomic Feature	CpG Island Association	DMR	Log2 Ratio	FDR	R^2
FAM86JP	Promoter	Island/North Shore	N/A	0.2012	47.43	0.2884
TTC7B	Promoter	South Shore	N/A	-0.2192	47.25	0.5194
FAM86C2P	Promoter / Gene Body	Island	N/A	0.2310	46.89	0.2156
GRIN1	Gene Body	Island/North Shore	N/A	-0.3017	46.65	0.2898
LFNG	Gene Body	South Shore	N/A	-0.3641	46.65	0.1898
N/A	N/A	HMM Island	N/A	0.2835	46.65	0.3944
N/A	N/A	North Shore	RDMR	-0.3885	46.65	0.5595
SOHLH1	Promoter / Gene Body	Island/North Shore	N/A	-0.2081	46.39	0.1542
N/A	N/A	South Shore	RDMR	-0.3423	46.34	0.1679
N/A	N/A	Island/North Shore	N/A	-0.2100	46.34	0.3924
SLC22A18AS	Gene Body	South Shore	N/A	-0.2397	46.34	0.5081
PURA	Promoter	Island/North Shore	N/A	-0.2042	46.08	0.4237
NFAT5	Promoter	North Shore	RDMR	-0.2129	46.05	0.1748
DMPK	Gene Body	Island	N/A	-0.3335	46.04	0.2442
LOC100133461	Promoter	North Shelf	N/A	-0.4967	46.04	0.3899
N/A	N/A	Island/North Shore	CDMR	-0.2369	46.04	0.4311
N/A	N/A	HMM Island	N/A	-0.3640	46.04	0.2529
PTPRN2_2	Gene Body	Island/North Shore	N/A	-0.2666	46.04	0.1169
PITX1	Gene Body	North Shore	CDMR	-0.2952	45.96	0.1888
ARHGEF10	Gene Body	N/A	N/A	-0.3564	45.72	0.2585
N/A	N/A	North Shore	N/A	-0.7087	45.72	0.222
PALM	Gene Body	Island	N/A	-0.2109	45.72	0.3631
C7orf50	Gene Body	North Shore	N/A	-0.2133	45.54	0.1568
SEMA6B	Gene Body	Island/North Shore	CDMR	-0.3163	45.39	0.3574
FOXK1	Gene Body	South Shore	RDMR	-0.4457	45.27	0.4838
FAM86C1	Promoter / Gene Body	Island	N/A	0.2260	45.18	0.1453

Table 4.1: Continued

Gene	Genomic Feature	CpG Island Association	DMR	Log2 Ratio	FDR	R^2
ADAMTS8	Promoter	South Shore	N/A	-0.2193	44.74	0.5308
N/A	N/A	North Shore	N/A	-0.2771	44.67	0.2686
EDARADD	Promoter	North Shore	N/A	-0.2506	44.52	0.3686
FAM86B2	Promoter	Island	N/A	0.2238	44.48	0.2209
AGRN	Promoter	South Shore	N/A	-0.5087	44.46	0.3049
LEMD2	Promoter	North Shore	N/A	-0.2055	44.46	0.414
MTMR8	Promoter / Gene Body	Island/North Shore	N/A	0.2070	44.27	0.3698
MIR9-3	Promoter	Island/North Shore	N/A	-0.2235	44.17	0.4838
KRT7	Promoter	North shore	N/A	-0.2041	44.15	0.276
NKX2	Promoter	Island/North Shore	RDMR	-0.3287	44.01	0.3185
N/A	N/A	North Shore	N/A	-0.2408	43.86	0.3225
N/A	N/A	North Shore	RDMR	-0.3785	43.86	0.6517
N/A	N/A	North Shore	RDMR	-0.3876	43.56	0.3218
USP6NL	Gene Body	Island	N/A	-0.4037	43.54	0.1384
N/A	Promoter	North Shore	N/A	-0.2067	43.22	0.3973
N/A	N/A	Island	N/A	-0.2748	42.66	0.5203
NBLA00301	Gene Body	North Shore	RDMR	-0.2964	42.35	0.5779
AJAP1	Gene Body	South Shore	RDMR	-0.3908	42.06	0.1215
CRYBA2	Gene Body	North Shore	N/A	-0.2093	42.06	0.587
CTF1	Promoter	South Shore	N/A	-0.2488	42.06	0.501
FOXF2	Gene Body	South Shore	RDMR CDMR	-0.2036	41.96	0.3976
MAP4K1	Promoter	North Shore	N/A	-0.2117	41.91	0.3082
N/A	N/A	HMM Island	N/A	-0.2422	41.86	0.2107
BCL11A	Gene Body	N/A	N/A	0.2415	41.79	0.2955
N/A	N/A	North Shore	RDMR	-0.2307	41.76	0.529
LONP1	Gene Body	Island	N/A	-0.2769	41.19	0.3134
N/A	N/A	HMM Island	N/A	-0.2885	41.19	0.3396
TBC1D10A	Gene Body	North Shore	N/A	-0.3085	41.19	0.528
CALCA	Gene Body	North Shore	N/A	-0.2781	40.89	0.2362
DNMT3B	Gene Body	South Shore	RDMR	-0.3683	40.89	0.2687
VAX2	Gene Body	North Shore	RDMR	-0.2485	40.89	0.3199
ZFPM1	Gene Body	Island	N/A	-0.2848	40.76	0.1458

Table 4.1: Continued

Gene	Genomic Feature	CpG Island Association	DMR	Log2 Ratio	FDR	R^2
OXLD1	Gene Body	North Shore	N/A	-0.2737	40.60	0.3644
FSCN1	Gene Body	South Shore	RDMR	-0.3639	40.31	0.3546
FXYD6	Promoter	South Shore	N/A	-0.3141	40.31	0.2952
NADK	Promoter	South Shore	RDMR	-0.2196	40.31	0.3951
PARP12	Gene Body	North Shore	CDMR	-0.2035	40.31	0.3821
TBX5	Promoter / Gene Body	Island/North Shore	N/A	-0.2904	40.13	0.3641

The average called window is approximately 887 base pairs in length and contains an average of 5 CpGs with no fewer than 3 in any significant window. Of the 139 hypomethylated regions, 112 are associated with a gene (at either the promoter or the gene body) and of the 8 hypermethylated regions, 7 are gene associated. In one case, we identified 3 significantly hypomethylated windows within a single gene (PTPRN2). Thus, there were a total of 110 genes with age-associated hypomethylation.

The significant loci identified in our analyses are located at various genomic features. The majority of regions that displayed age-associated hypomethylation events occur at CpG shores and not in CpG islands themselves, whereas hypermethylation events are more commonly associated with CpG islands (Figure 4.2A-B). In most cases, age-associated methylation alterations occur at regions that likely impact gene transcription (gene bodies, promoters). However, our data also indicate that these alterations are relatively subtle, with hypomethylation events displaying an average intra-individual % methylation decrease (calculated as a β-value for array data with values between 0 and 1) of approximately 0.039, ranging from 0.01 to 0.104 between paired samples (young and aged). Similarly, for age-associated hypermethylation events, the average % methylation increase was approximately 0.032 (Figure 4.2C). Importantly, these alterations all occur in windows with an average initial % methylation of <0.6 at the first collection and the majority (68% of hypomethylation events and 50% of hypermethylation events) are also considered to have intermediate methylation based on conventional standards (β-value between 0.2 and 0.8).

We additionally analyzed the colocalization of windows of age associated methylation alterations with known regions of nucleosome retention in the mature sperm,

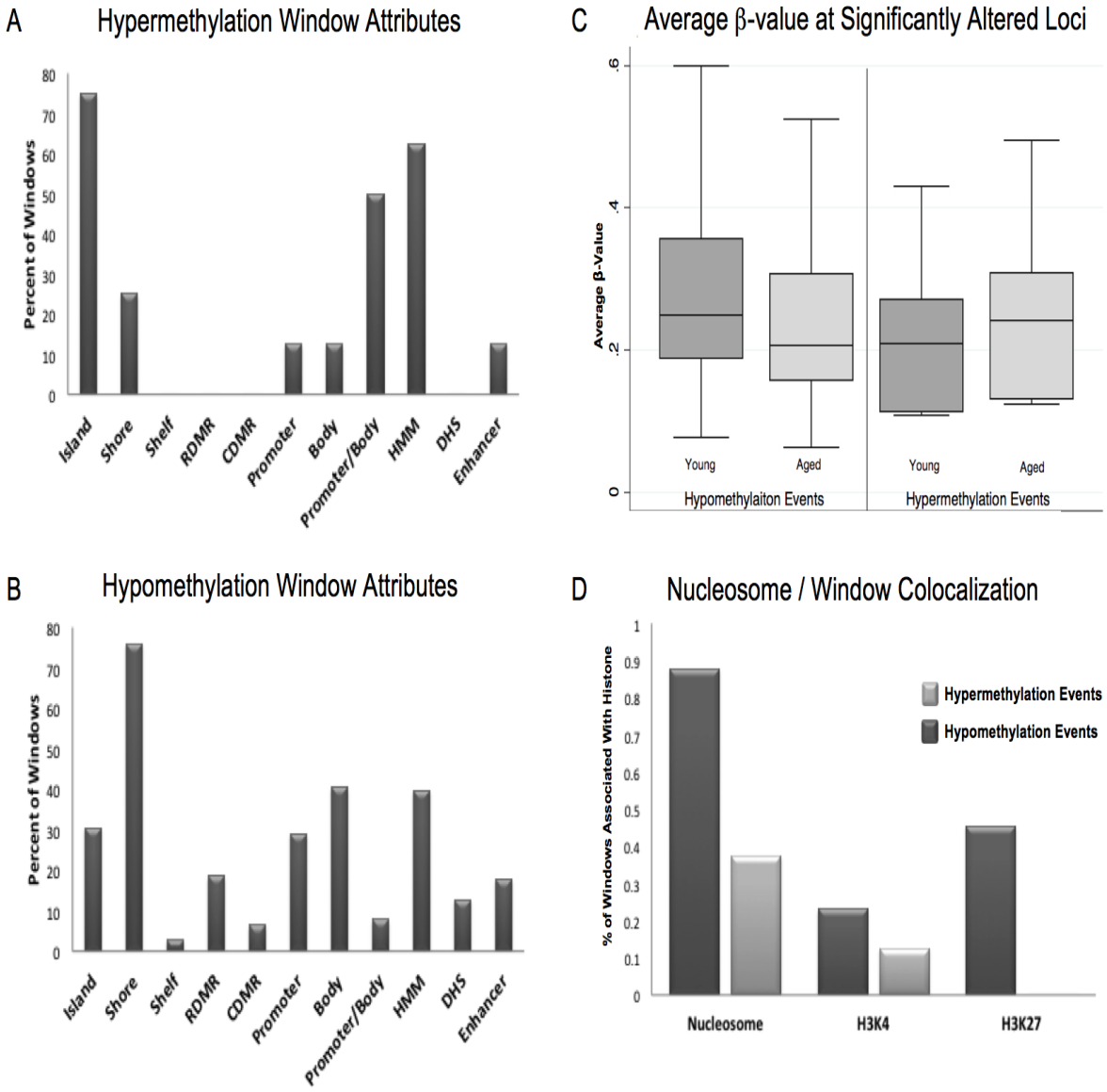

Figure 4.2: The attributes of significant windows identified in our study for both hypermethylation events and hypomethylation events (A and B, respectively). These designations are based on UCSC annotation at the regions of interest. Average β-values for all significant windows (hypomethylation and hypermethylation events) for both aged and young (C). Average decrease in β-value for intra-individual hypomethylation events was approximately 3.9% and for hypermethylation events was 3.2%. We also investigated the colocalization of nucleosomes (every region of known histone retention) as well as histone modifications (H3K4 methylation, and H3K27 methylation) with our windows of interest (D). Hypermethylation events were less frequently associated with all retained histones (nucleosomes) and loci with H3K27 methylation when compared to hypomethylation events based on Fisher's Exact Test ($p=0.002$; $p=0.0107$). Co-localization of hypermethylation or hypomethylation events with H3K4 methylation was statistically similar.

as well as regions where specific histone modifications are found based on previous work from our laboratory [12]. We found that approximately 88% of regions that are hypomethylated with age are found within 1 kb of known nucleosome retention sites in the mature sperm (Figure 4.2D.). Interestingly, loci that are hypermethylated with age are far less frequently found in regions of histone retention, with only approximately 37.5% being associated with sites where nucleosomes are found. This difference was significant based on a Fisher's Exact test. Similarly, 23% of loci with age-associated hypomethylation are associated with H3K4 methylation and 45.3% are associated with H3K27 methylation. The same colocalization is very rare with hypermethylaiton events. Additionally, we analyzed chromosomal enrichment of these significant marks to determine if there are specific chromosomal regions that are more susceptible to age-related methylation alterations. We found a random distribution of significant age-associated methylation alterations throughout the entire genome with no one chromosomal region being significantly enriched (Figure 4.3.).

Sequencing Analysis

A representative group of windows was selected for targeted sequencing to confirm that the CpGs tiled on the array were representative of the entire CpG content within the windows of interest. Both young and aged samples from each donor were analyzed and compared at these specific loci. In 20 of the 21 gene regions that were analyzed, the array and MiSeq data were similar in both direction and relative magnitude (Figure 4.4.). In the one case that did not show a similar trend (hypomethylation with age by array and no change by MiSeq), the amplicon was outside the region of the two CpGs

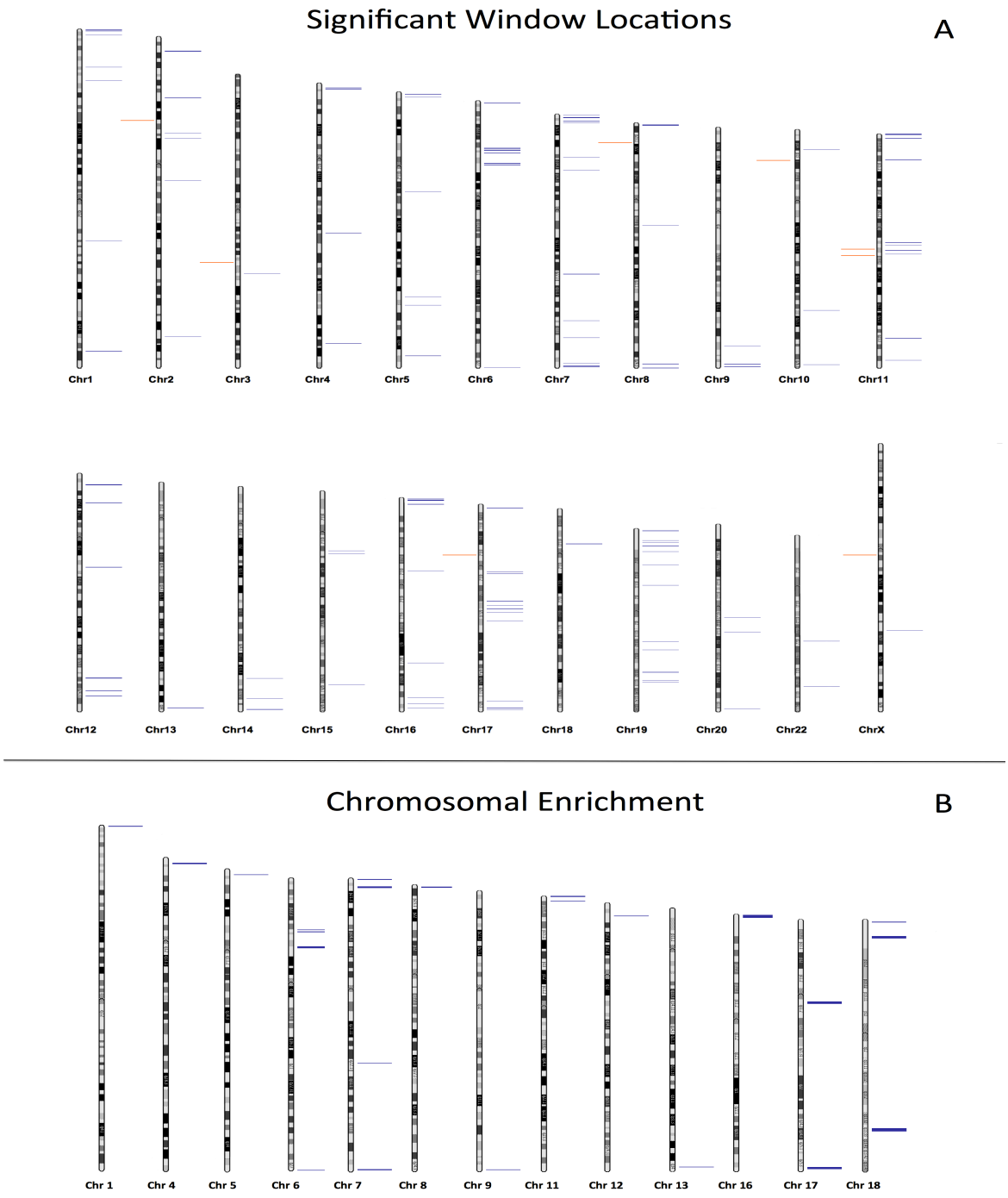

Figure 4.3: Chromosomal loci of each altered region are depicted where blue marks are hypomethylation events and red marks are hypomethylation events (A). The Correlation Maps app on the USeq platform was used to locate any specific chromosomal enrichment of altered methyaliton windows. Specifically, the application called any 100kb region where at least two significantly altered methylation marks were found. All called chromosomal enrichment regions are displayed (B) though none were found to be significantly enriched over the background.

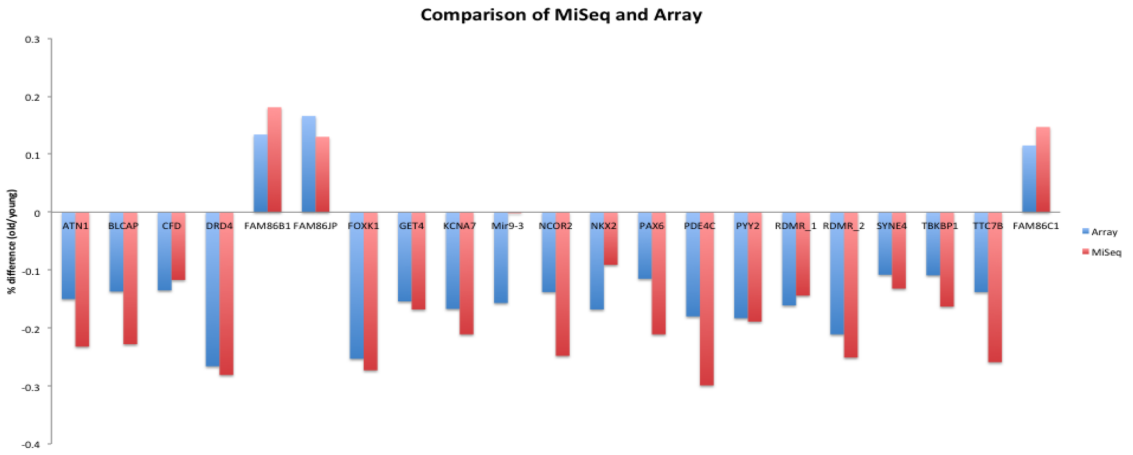

Figure 4.4: Comparison of MiSeq results to our array results at 21 representative regions. Because beta-values and fraction methylation are generated in a different manner (array vs. sequencing, respectively) they are not directly comparable. For this reason, we compared the % difference for each loci and each technology. This is accomplished by the following equation: % difference = (aged value/young value) – 1.

that drove the significance of the window. When comparing the methylation of the approximately 300bp amplicon to the CpG tiled on the array in that same region only, and not the array CpGs over the entire 1000bp window, the data are in agreement. Taken together, the sequencing run confirmed that our array data are a good representation of the methylation status at all CpGs in our regions of interest.

GO Term Pathway and Disease Association Analysis

The genes affected by the age-associated methylation alterations (those that have alterations that occur at their promoter, or gene body) were analyzed by Pathway, GO, and disease association analysis. The results indicate that no one GO term or Pathway is significantly altered in our gene group. Similarly, there were no significant diseases or disease classes associated with the genes identified in this study based on results of the disease association tool on DAVID. However, the most significant disease hits (those that

were significant prior to multiple comparison correction) have both been suggested to have an increased incidence in the offspring of older fathers, namely myotonic dystrophy and schizophrenia [2,7]. To directly investigate the disease association(s) in our set of genes, we searched the National Institute of Health's (NIH) genetic association database (GAD), which is utilized in DAVID's disease association analysis algorithm. We investigated all 117 genes that were determined to have age-associated methylation alterations (110 hypomethylated; 7 hypermethylated) for their various disease associations. A total of 46 genes from our group have been confirmed to be associated with either a phenotypic alteration or a disease based on GAD annotation. We identified 4 diseases that had known associations with at least 3 of our genes (diabetes mellitus, hypertension, bipolar disorder, and schizophrenia). To further investigate these associations, we analyzed the frequency of genes associated with these 4 diseases in our gene set and compared it to their frequency in all 11,306 genes known to be associated with either a phenotypic alteration or a disease. This analysis revealed that both bipolar disorder and schizophrenia were more frequently associated with regions prone to age-related methylation change than the background set of genes based on Fisher's Exact test ($p = 0.001$ and $p = 0.005$, respectively; Figure 4.5). The frequency of genetic association between our gene set and the background gene set was statistically similar for both hypertension and diabetes mellitus.

Discussion

Herein we report alterations to sperm DNA methylation associated with age. Interestingly, our data are in contrast with previous reports of age-associated methylation

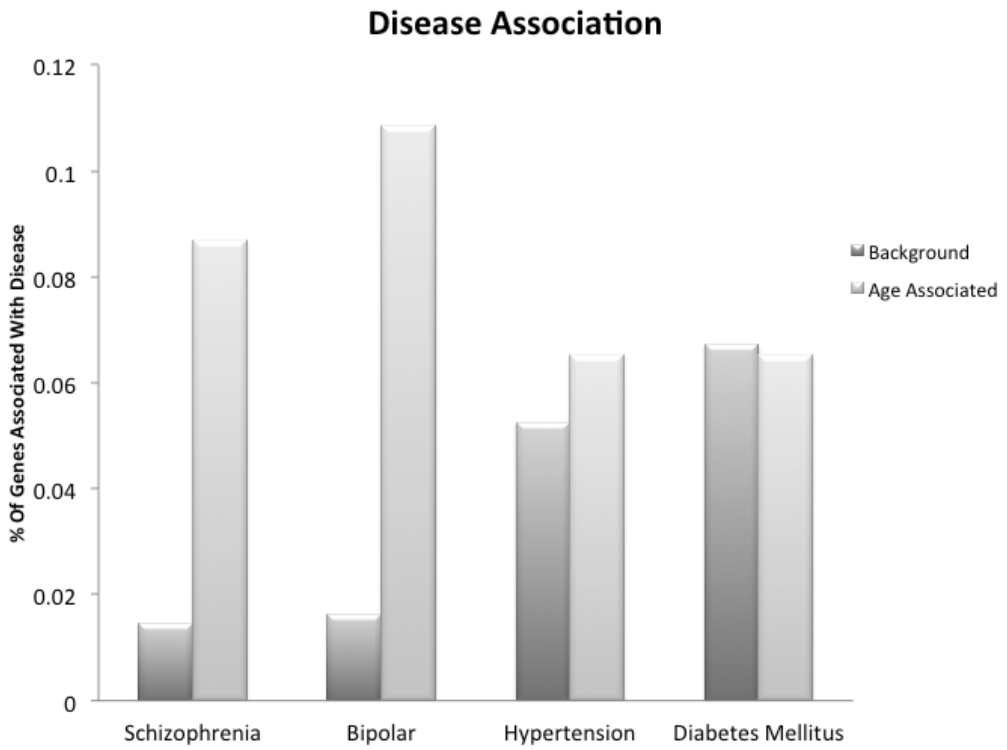

Figure 4.5: The frequency of disease associations within our gene set was analyzed and compared to the frequency of disease associations for all genes known to be associated with at least a single disease based on GAD annotation. Schizophrenia, bipolar disorder, diabetes mellitus, and hypertension were selected as there were at least 3 genes in our small set of identified genes that are associated with these diseases. Only bipolar disorder and schizophrenia were more frequently associated with our identified genes then the background set of genes based on Fisher's Exact test with p-values of 0.001 and 0.005 respectively. The frequency of genes associated with hypertension and diabetes mellitus in the two groups was statistically similar.

alterations in somatic cells. Recent literature suggests age-associated global hypomethylation with regional (gene associated) hypermethylation in somatic tissue [18]. In contrast, our data reveal age-associated hypermethylation globally with a strong bias toward hypomethylation regionally. This is less surprising when we take into account the fact that sperm are known to have other age-associated modifications that defy convention (i.e., telomere length) [19-21]. Intriguingly, while the methylation alterations reported herein are relatively subtle, they are strikingly significant and are common

among individuals at various ages and intervals between collections, suggesting that these regions are consistently altered over time in a linear fashion. Importantly, it appears that many significantly altered regions are at loci that may contribute to various diseases known to have increased incidence in the offspring of older fathers. Coupling these with our data demonstrating that no one GO term or pathway is up- or down-regulated in the sperm as a result of the aging process suggests that the alterations we observed are the result of regional genomic susceptibility to methylation alteration. This hypothesis also comports well with the linear nature of the alterations we observed. While the nature of this susceptibility is difficult to elucidate, it may be related to chromatin architecture.

Localization of Altered Regions

To investigate the attributes of regions that we determined to be most susceptible to methylation alterations, we evaluated the colocalization of significantly altered loci in our study with regions of nucleosome retention in the mature sperm. It appears that hypomethylation events are most commonly associated with sites of nucleosome retention. Interestingly, this same colocalization was not seen with hypermethylation events. Though colocalization patterns are significantly different between the hypomethylation and hypermethylation events, it should be noted that the sample size is quite small in the hypermethylation group (8 significant windows). It should also be noted that while the colocalization of histones and the hypomethylation events we observed in our study are significant, the methylation marks observed are likely established earlier in spermatogenesis and thus may not be affected by the nucleosome architecture in the fully matured sperm. In addition, the alterations identified in this study are not localized everywhere that histones are retained; thus, nucleosome retention alone

cannot be the independent driving force of regional susceptibility to methylation alterations.

Recent literature suggests an interesting hypothesis of "selfish spermatogonial selection" that may have application in this study as well [22]. Briefly, the hypothesis states that some gene mutations that are causative of abnormalities in the offspring are beneficial to spermatogenesis and become enriched throughout the aging process in spermatogonial stem cells. Thus, sperm carrying these mutations become more frequent in the population to the detriment of the offspring. Similarly, it is possible that the age-associated methylation alterations we have identified may be in regions that are important to spermatogenesis and thus would be selected for. The hypomethylation events that are selected for could occur as a result of either active or passive demethylation. For example, regional transcription activity at loci important in spermatogenesis would likely be accompanied by a relaxed chromatin structure that could result in increased frequency of DNA damage over time. Established methylation marks located within this region could then be passively removed through repair mechanisms in the developing sperm. If the removal of this mark is either beneficial or has no effect on spermatogenesis, it will persist, and over time, similar marks could accumulate at nearby CpGs, ultimately leading to the profile we identified in our study. Alternatively, active enzymatic removal of methylation marks in the sperm might drive age-associated methylation changes. For this to be mechanistically plausible, we would have to assume that hypomethylation in the windows we identified is always beneficial to spermatogenesis. While either of these mechanisms is plausible, it is likely that the effects we have identified involve some combination of both.

The mechanics of hypermethylation events with age are more difficult to elucidate, as this, by definition, has to be an active targeted process involving methyltransferase enzymes. However, some evidence from this study indicates DNA sequence may be an important driver of age-related hypermethylation. Of the 7 windows that we identified with gene-associated hypermethylation with age, 4 are associated with the FAM86 family of genes that are categorized not by protein function or genomic location but sequence similarity. This strongly suggests that, at least in part, age-associated hypermethylation events at specific loci are driven, either directly or indirectly, by DNA sequence. Interestingly, this family of genes (FAM86) with unknown function has recently been categorized with a larger family of methyltransferase genes, though it remains unclear what the FAM86 target(s) is/are (DNA, Histone, other proteins, etc.). Taken together, the mechanisms that drive age-related methylation alterations in the sperm remain elusive, but likely involve both active and passive methylation modification.

Biological Significance

It is important to consider two questions in determining the biological impacts of the identified methylation changes in this study. First, are the methylation changes described herein capable of transcriptional alterations? Second, are these methylation changes capable of avoiding embryonic methylation reprogramming? Regardless of the mechanism by which these methylation marks are altered in the sperm over time, it is striking that these changes occur with such consistency between individuals and have such a tight association with age. One limitation of these findings, however, is the magnitude of alterations we have discovered. As described earlier, the average intra-

individual alteration at any given window was approximately a change of 3.9%. Though this seems relatively small, when expanded to include the possible reproductive years in a male (approximately age 20-60), the change would be 10-12%. Importantly, because of the heterologous nature of the sperm population, a change of this magnitude in average β-value over a window including multiple CpGs needs to be considered in two different ways. First, a decrease of 10-12% reflects a complete methylation erasure (from fully methylated to fully demethylated at all CpGs within a given window) in 10-12% of the sperm population. The second possibility is that the observed β-value alterations reflect changes to random CpGs within windows of susceptibility in all sperm, which would manifest as hemimethylation in the region of interest in all sperm in the population. The resultant 10-12 % change in methylation within every individual sperm (effectively 1 out of every 10 CpGs are demethylated) suggests that every sperm carries similar, more subtle, alterations within these windows on average. It is likely that the degree and distribution of these alterations throughout the entire sperm population varies greatly depending on the region of interest and the demethylation process (active or passive). The resultant epigenetic landscape alterations in either case may contribute to disease susceptibility in the offspring despite the small degree of change across the whole population though the increased risk to the offspring may be relatively small. Figure 4.5 gives a breakdown the alterations seen at two representative loci, DRD4 and TNXB.

The heritability of such marks is more difficult to elucidate mainly because the current study does not directly address this question. However, this issue needs to be addressed as the identified age-associated methylation alterations in the mature sperm may be removed through the embryonic demethylation wave. It should be noted that

despite the fact that there is no direct evidence of methylation alteration heritability at the specific loci presented in this work, the observed age-associated changes at regions known to be of significance in diseases with increased incidence in the offspring of aged males is striking and warrants further study. The intriguing localization of these alterations suggests that the methylation profile in the mature sperm, at specific loci, either contributes to the increased incidence of associated abnormalities in the offspring or that they reflect (are downstream of) changes that are actually causative of the associated abnormalities in the offspring. Moreover, it has been previously proposed that epigenetic alterations are among the most likely candidates to transmit such transgenerational effects, and we have identified methylation alterations that appear capable of contributing to the various pathologies associated with advanced paternal age. Despite this, future work must still be performed to determine the real impact these marks have on transcription and thus phenotype and disease. Taken together, these subtle yet highly significant, age-associated alterations to the sperm methylation profile are intriguing because of their location and consistency, but more work is required to elucidate the biological impact of these marks.

There are many genes identified in our study that, if biologically affected, may result in pathologies in the offspring. Dopamine receptor D4 (DRD4) is one of the most influential genes in the pathology of both schizophrenia and bipolar disorder as well as many other neuropsychiatric disorders [23,24]. Interestingly, the entire DRD4 gene itself is strongly hypomethylated with age (Figure 4.6). TNXB has also been suggested to be associated with schizophrenia based on multiple studies, though the data are controversial [25,26], and virtually the entire 1st exon of TNXB is hypomethylated with age. Additionally,

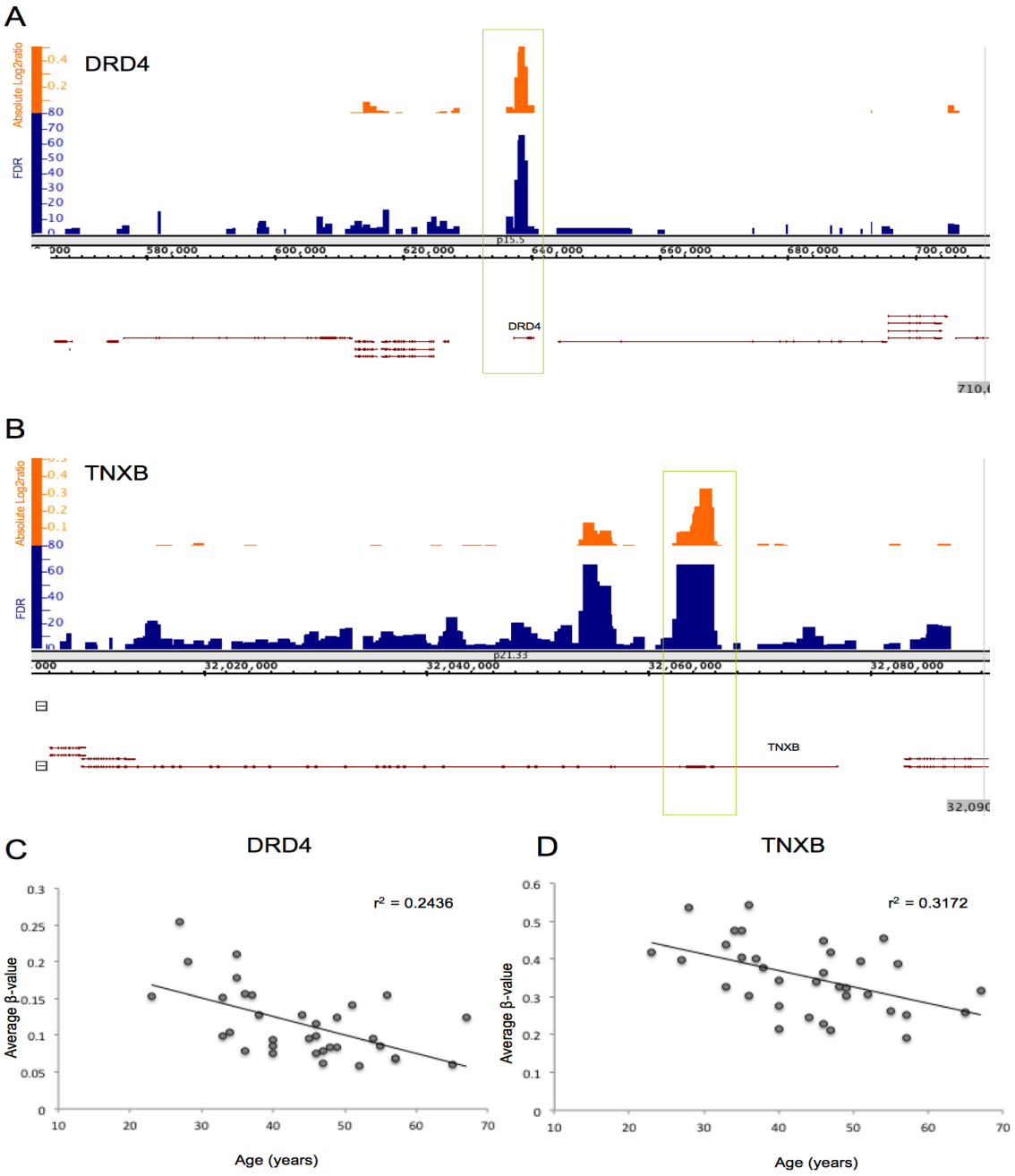

Figure 4.6: Descriptive statistics are presented for both TNXB and DRD4; 2 regions of representative methylation alterations. The alignment track for each gene is displayed in Integrated Genome Browser (IGB) with the associated false discovery rate (FDR) denoting the significance of the change and the and absolute log 2 ratio reflecting the magnitude of the alteration (A,B). Scatter plots for each sample from all 17 donors (a total of 34 samples with each donor represented twice) revealed a significant decrease in methyalation with age at both DRD4 and TNXB (p=0.0005 and p=0.003, respectively; C,D).

DMPK, a gene identified in our study, is known to be associated with myotonic dystrophy, a disease for which advanced paternal age is a risk factor [7]. In fact, increases in trinucleotide repeats in DMPK are believed to be the cause of myotonic dystrophy type 1. Importantly, previous data suggest that altered methylation marks may affect trinucleotide instability [27]. These examples represent only a portion of the genes that were identified in our study and support the hypothesis that age-associated DNA methylation alterations in sperm may play a role in the etiology of various diseases associated with advanced paternal age.

Future Directions

There are 2 important findings in this study. First, that there are any age-associated alterations common among such a varied study population is remarkable. Specifically, age-associated methylation alterations occur in the sperm regardless of whether the ages between collections are approximately 20 to 30 years of age or 50 to 60 years of age. Second, the increased frequency of genes associated with bipolar disorder and schizophrenia in our study when compared to all genes associated with disease provides intriguing insight into the increased susceptibility of these specific disorders in the offspring of older fathers. Though frequently hypothesized, this work comprises, to the best of our knowledge, the first direct evidence suggesting the plausibility of epigenetic alterations in the sperm of aged fathers influencing, or even causing, disease in the offspring. Because of the nature of the unique sample set we have utilized in this study, future work is needed to directly address a number of questions. Are methylation alterations, similar to those seen in our study, causative of neuropsychiatric disease? Can the methylation marks we observe in our study avoid embryonic demethylation? Future

targeted work is required to directly address these questions to enable us to determine the role that these altered methylation marks play in the increased incidence of various diseases seen in the offspring of older fathers.

Methods

Study Group

Under an Institutional Review Board approved study, our laboratory has accessed samples from 17 sperm donors (of known fertility) that were collected in the 1990s. These donors provided an additional semen sample in 2008, enabling the evaluation of intraindividual changes to sperm DNA methylation with age. These samples are referred to as young (1990s collection) and aged (2008 collection) samples. The age difference between each collection varied between 9 and 19 years, and the age at first collection ("young" sample) was between 23 and 56 years of age.

At every collection, donors were required to strictly follow the University of Utah Andrology Laboratory collection instructions, which includes abstinence time of between 2 and 5 days. The whole ejaculate (no sperm selection method was employed) collected at each visit was frozen in a 1:1 ratio with Test Yolk Buffer (TYB; Irvine Scientific, Irvine, CA) and stored in liquid nitrogen prior to DNA isolation. Samples were thawed and the DNA was extracted simultaneously to decrease batch effects. Prior to DNA extraction, samples underwent somatic cell lysis by incubation in a somatic cell lysis buffer (0.1% SDS, 0.5% Triton X-100 in DEPC H_2O) for 20 min on ice to eliminate somatic cell contamination. Samples were visually inspected following lysis to ensure the absence of all potentially contaminating cells before proceeding. Following somatic cell lysis, sperm DNA was extracted with the use of a sperm-specific extraction protocol used

routinely in our laboratory [28]. Briefly, sperm DNA was isolated by enzymatic and detergent-based lysis followed by treatment with RNase and finally DNA precipitation using isopropanol and salt, with subsequent DNA cleanup using ethanol.

Microarray Analysis

Each of the paired samples for the 17 donors (a total of 34 samples) were subjected to array analysis using the Infinium HumanMethylation 450 Bead Chip micro-array (Illumina, San Diego CA). Extracted sperm DNA was bisulfite converted with EZ-96 DNA Methylation-Gold kit (Zymo Research, Irvine CA) according to manufacturer's recommendations. Converted DNA was then hybridized to the array and analyzed according to Illumina protocols at the University of Utah genomics core facility. Once scanned and analyzed for methylation levels at each CpG, a ß-value was generated by applying the average methylated and unmethylated intensities at each CpG using the calculation: ß-value = methylated/(methylated + unmethylated). The resultant ß-value ranges from 0 to 1 and indicates the relative levels of methylation at each CpG with highly methylated sites scoring close to 1 and unmethylated sites scoring close to 0.

Basic descriptive analyses of the microarray data were performed using Partek (St. Louis MO). More in-depth analysis was performed using the USeq platform with the application Methylation Array Scanner which identifies regions of altered methylation that are common among individuals utilizing a sliding window analysis. Briefly, paired data from each donor (young and aged) were subjected to a 1000 base pair sliding window analysis where regions of altered methylation with age that are common among donors were called by Wilcoxon Signed Rank Test. To prevent the influence of outliers in the data set, methylation for a specific window was reported as a pseudo-median and

differences between the young and aged sample are reported as log2 ratios. Two thresholds were applied to identify windows with significant differential methylation. A Benjamini Hochberg corrected Wilcoxon Signed Rank Test FDR of >=0.0004 and an absolute log2 ratio >= 0.2. To confirm the significance of each of the called windows, we subjected the mean β-value within the window for each donor (young and aged samples) to a paired t-test. Following this initial filter, we additionally subjected each significant window to a regression analysis to determine the relationship between age and mean methylation within each window. Regression analysis and paired t-tests were performed using STATA 11 software package.

Sequencing Analysis

Each sample was additionally subjected to targeted methylation sequencing at loci determined to be of interest based on microarray analysis. This step was designed to confirm the array results and to provide greater depth of coverage of the CpGs in the windows of interest. Primers for 21 loci were designed using MethPrimer (Li Lab, UCSF). PCR was performed on samples following sperm DNA bisulfite conversion with EZ-96 DNA Methylation-Gold kit (Zymo Research, Irvine CA). PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia CA) and were pooled for each sample. The Pooled products were delivered to the Microarray and Genomic Analysis core facility at the University of Utah for library prep which included shearing of the DNA with a Covaris sonicator to generate products of approximately 300 base pairs, in preparation for 150 bp paired end sequencing, and the addition of sample-specific barcodes for all 34 samples. Multiplex sequencing was then performed on a single lane on the MiSeq platform (Illumina, San Diego CA).

Pyrosequencing Analysis

Each sample was subjected to pyrosequencing analysis of a portion of the LINE-1 repeatable element for the purpose of confirming previously determined global methylation changes with age. Briefly, isolated sperm DNA samples were submitted to EpigenDx (Hopkinton, MA) for the pyrosequencing analysis. Qiagen's PyroMark LINE1 kit was used to determine methylation status at each CpG investigated with the assay. The experiment was performed based on manufacturer recommendations.

GO Term / Pathway / Disease Association Analysis

GO term Analysis was performed with Gene Ontology Enrichment Analysis and Visualization Tool (GOrilla; cbl-gorilla.cs.technion.ac.il). Pathway and disease association analysis was performed on the Database of Annotation, Visualization, and Integrated Discovery (DAVID; david.abcc.ncifcrf.gov) v6.7. Additional disease association analysis was performed directly on the National Institute of Health's Genetic Association Database (GAD; geneticassociationdb.nih.gov).

Additional Statistical Analyses

Fishers Exact test was used to determine the differences in frequencies of genes associated with particular diseases between our significant gene group and a background group. This analysis was also used to detect the differences in frequencies of windows that were found in regions of histone retention in the hypomethylation group and the hypermethylation group. Additionally, regression analysis was utilized to determine relationships between age and methylation status at various loci. STATA software

package was used to test for significance with a $p<0.05$ being considered a significant finding.

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CHAPTER 5

CONCLUSIONS

Herein we have described the first direct evidence that regional age-associated epigenetic alterations to the sperm provide a plausible, though not yet fully elucidated, mechanism for the increased incidence of neuropsychiatric disorders in the offspring of older fathers. We will further discuss the social and biological implications of the "paternal age effect" and the contribution of the sperm epigenome to this effect, as well as the possible clinical significance of age-associated epigenetic alterations and important future directions that may, one day, facilitate the development of epigenetic diagnostic testing to provide helpful insight for couples of advanced reproductive age in their family planning decisions.

The Paternal Age Effect: Role of the Sperm Epigenome

There is a striking trend of delayed parenthood in developed countries, likely as a result of socioeconomic and secular pressures as well as increased rates of divorce and remarriage [1,2]. This trend is justified by increasing life expectancies; however, this delay may have undesirable impacts on fertility, pregnancy outcome, and even offspring health. There are very well-established negative consequences associated with advanced maternal age and fertility, pregnancy outcomes, and offspring abnormalities while the issue of male age has long been considered less significant. However, recent evidence has identified substantial negative impacts of advanced paternal age, some of which become

more pronounced when coupled with a female partner of advanced reproductive years. Specifically, as outlined earlier, advanced paternal age is associated with increased incidence of neuropsychiatric disorders, trinucleotide repeat associated diseases and some forms of nervous system and hematological cancers [3-13].

This is particularly troubling when considering that the trend of increased paternal age at conception continues to increase with no indication of reversal and that some of the undesirable effects may extend, or even be amplified, over multiple generations [14]. Intriguingly, concomitant with increasing delays in parenthood is increased incidence of neuropsychiatric disease [15]. Specifically, data presented from multiple groups at a recent conference in Switzerland all demonstrated increased incidence of psychoses and/or schizophrenia in Australian, Canadian, and English populations [15]. While these data remain controversial, the apparent trend of increasing cases of neuropsychiatric disorders taken together with the data demonstrating increased risk of developing these in the offspring of older fathers and the trend of delayed parenthood supports the growing epidemiological data suggesting an association between the age of the father and the likelihood of neuropsychiatric disease in the offspring.

While it is known that the incidence of these various disorders is associated with advanced paternal age, the actual mechanism or, more likely, mechanisms that drive this process are poorly understood and in many cases remain entirely elusive. Interestingly, many have hypothesized that epigenetic modifications, in particular DNA methylation alterations, are capable of driving such subtle changes in the father's germ cells that ultimately may lead to altered phenotype in the offspring. Our array and sequencing data demonstrate that the sperm epigenome is, in fact, significantly altered with age in a

linear, and relatively predictable, fashion at very consistent loci. This suggests that some regions of the DNA are more susceptible to methylation alterations than are others and that genes or regulatory features that lie in these regions are, in turn, more susceptible to age-associated transcriptional changes resultant from epigenetic modifications that may then contribute to the increased incidence of abnormalities in the offspring. This finding is remarkable because of its consistency between individuals throughout varied periods of aging and may prove to be very important to our understanding of offspring phenotypic alterations, and the study of transgenerational inheritance.

It is important to note that for the alterations we have discovered to have impact on the offspring, these aberrant methylation marks would have to escape embryonic methylation erasure and reestablishment. If these altered regions are capable of such demethylation avoidance and are able to persist and contribute to offspring abnormalities, then it is likely the alterations described herein may have effect beyond a single generation. In brief, if the altered marks are heritable and escape embryonic reprogramming, then they may accumulate over multiple generations, further compounding the issue of the paternal age effect. Indeed, available data suggest that the effect of advanced paternal age may have impact on grandchildren and not just the offspring of older fathers [5,14]. Thus, while the alterations we have detected are relatively subtle, it is easy, by extrapolating these effects over multiple generations, to understand that the negative impacts of advanced paternal age may be significantly compounded over time.

It is clear that advanced paternal age does have an impact on the offspring, though this impact may be subtle and only involve an increased risk of specific diseases. From

our data, it also appears that the described impacts on offspring phenotype may be mediated by epigenetic alterations, specifically DNA methylation changes in discrete genomic windows. While not definitive, these data provide us with strong evidence suggesting that sperm DNA methylation alterations that accumulate with age may impact offspring phenotype.

Possible Clinical Implications

While the study of age-associated sperm epigenetic alterations and their contribution to altered offspring phenotype is in its infancy, there are possible future clinical applications.

Diagnostic Testing

Epigenetic diagnostic tests on sperm DNA is difficult for many reasons. In terms of the future offspring, the most relevant and important information cannot be directly determined, specifically the epigenetic profile of the single sperm that fertilizes the egg. While it is not a possible to analyze this one cell, an understanding of the entire sperm population's epigenetic profile can inform patients of increased risks, which may be of value particularly for older patients where increased risks are more likely.

Altered methylation marks in the sperm that either contribute to, or are causative of, phenotypic alterations in the offspring cannot be treated, as targeted epigenetic therapy is currently unrealistic. However, it is not unreasonable to imagine epigenetic diagnostic testing on the germ cells of the father, probing regions that are known to be altered with age in sperm DNA and that are causative of, or contribute to, offspring phenotype alterations. Such testing would inform couples considering having children at

an advanced reproductive age of possible increased risks. It is likely, and our data suggest, that at some loci known to undergo age-associated epigenetic alteration, the magnitude of changes varies significantly between individuals, possibly a result of lifestyle differences (diet, exercise, tobacco/alcohol use, etc.). Thus, while all aged individuals may have an increased risk of siring offspring with the previously described disorders, some may have a particularly high risk compared to others and this information could aid patients in making important family planning decisions.

Preemptive Measures

Recent media coverage of the paternal age effect on offspring has increased not only the scientific communities' interest in the topic, but also the general public's. It is important that accurate information regarding the risks of fathering children at an advanced age be presented to men early enough that they are able to make well-informed decisions. It is again important to note that while the increased relative risk associated with advanced age is quite striking, the absolute risk to the offspring remains fairly low. Regardless, many individuals would still prefer to avoid this risk if possible. With this in mind, there are a few possible interventions that can be and, in some cases, are already being explored by physicians and their patients.

Currently, there are a number of steps that can be taken by concerned individuals to avoid the possible negative consequences of fathering children at an advanced age. These options have varying degrees of effectiveness. Likely, the most effective method is simply starting families at an age earlier than individuals had previously anticipated through natural conception, though this may be unrealistic for many as a result of socioeconomic pressures. More frequently, individuals will likely choose other options,

such as cryopreservation of sperm at a young age, as a possible way to avoid the negative consequences of advanced age on the sperm. In fact, many private tissue banks now advertise such services. While this may be a beneficial, we have little data regarding the negative impacts of cryopreservation on the sperm and particularly on the offspring. Additionally, the reproductive therapies used to generate offspring with the cryopreserved samples may introduce unwanted risks as well. While there are little data to support this practice, sperm cryopreservation introduces little to no risk to the individual storing the sample and is relatively inexpensive. As a result, it is not unreasonable for a physician to explore such options, though the patients must be well informed of the risks and benefits.

Taken together, much more information is needed to increase our understanding the paternal age effect and the influence of the sperm epigenome in the process. With a more thorough understanding of these issues, we can better evaluate the benefit of various diagnostic tests or preemptive measures designed to decrease the risks of advanced paternal age and the impacts on offspring phenotype.

Future Directions

Much work is still required to gain a more complete understanding of the epigenetic alterations in sperm that are capable of phenotypic alterations in the offspring. We have identified a number of genomic regions that both undergo methylation alterations with age and are important in various diseases known to have increased incidence in the offspring of older males. To determine if these marks can contribute to disease susceptibility in the offspring, a number of unanswered questions must be addressed.

What is the impact of altered methylation profiles at our regions of interest? To completely understand the alterations we have seen and their impact on offspring phenotype, we have to determine if these alterations are associated with transcriptional changes. Future work can target either paired samples, as we did in our study, from multiple individuals (young and aged) or a large number of individuals of various ages and identify both the common methylation alterations that occur with age and the associated changes to transcriptional activity. Ideally, future work would also include offspring methylation status and transcriptional activity from multiple tissues in the offspring.

Do the altered methylation marks seen in sperm escape embryonic demethylation? To effectively accomplish this, an aged animal model should be used. Sperm from aged mice and young mice could be analyzed to confirm our findings (windows of altered methylation at analogous regions). The presence or absence of similar methylation marks in the offspring can then be determined. To increase the rigor of such a study, a highly polymorphic strain of mouse could be mated with a common background strain to assist in determining parent of origin, effectively enabling the researcher to follow changes on the paternal DNA with a great degree of confidence.

Do methylation perturbations contribute to neuropsychiatric disorders? Large epidemiological studies that identify epigenetic changes in very well-defined groups (bipolar disorder, schizophrenia, autism, etc.) are needed. These epigenome-wide studies have the capacity to identify both striking and subtle associations. The identification of even mildly altered genomic loci that are commonly perturbed in humans with specific diseases will enable us to better understand our data set and its importance to the

increased incidence of these diseases in the offspring of older fathers. Such a project was recently initiated for the study of Autism. Intriguingly, this specific study will utilize the 450K array as was used in our study; thus, our two data sets can be directly compared to determine any similar findings.

Conclusion

Our data represent, to the best of our knowledge, the first analysis of age-associated alterations to the human sperm methylome with both a global, regional, and CpG specific approach. While the alterations we have identified are subtle, they are strikingly consistent between individuals at very specific loci. As a result, the impact of these alterations may be of great significance to the study of transgenerational inheritance. A more complete knowledge of the true phenotypic impact of these methylation marks as well as their association with disease will be important to determine the role of age-associated sperm methylation alterations in the increased incidence of various diseases in the offspring of older fathers.

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